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(54) Title: SUBUNIT VACCINE AGAINST FLAVIVIRUS INFECTION

(57) Abstract

The invention provides vaccines capable of eliciting the production of neutralizing antibodies against flavivirus containing, as an active ingredient, a secreted recombinantly produced envelope protein or subunit thereof. In illustrative embodiments, the B domain or the 60 % or 80 % N-terminal portions of the envelope protein (E) of dengue virus are secreted. The secreted product can easily be purified and prepared as a vaccine.

SUBUNIT VACCINE AGAINST FLAVIVIRUS INFECTION

Technical Field

5   The invention relates to protection against and
diagnosis of the conditions caused by flavivirus. More
specifically, the invention concerns subunits of the
flavivirus envelope protein secreted as mature recombinantly
produced proteins from eucaryotic cells. Additional viral
10 proteins or subunits, also produced in this way, provide
additional active ingredients. These E-proteins or
subunits, alone or in combination including combination with
additional viral-derived peptides are protective against
infection by flavivirus, raise antibodies useful in passive
15 immunization, and are useful in diagnosis of infection by
the virus.

Background Art

      The flavivirus family, Flaviviridae, includes the
20 dengue viruses Japanese encephalitis (JEV) virus, Tick-borne
encephalitis (TBE) virus, and the initially discovered
prototype of this class, the yellow fever (YF) virus. The
flaviviruses contain a single positive strand genomic RNA
and are small enveloped viruses affecting animals, but
25 generally transmitted to vertebrates by chronically infected
mosquito or tick vectors. Flaviviruses are enveloped by
host cell membrane and contain the three structural proteins
capsid (C), membrane (M), and envelope (E). The E and M
proteins are found on the surface of the virion where they
30 are anchored in the membrane. Mature E is glycosylated,
whereas M is not, although its precursor, prM, is a
glycoprotein. Glycoprotein E, the largest structural
protein, contains functional domains responsible for cell
surface attachment and intraendosomal fusion activities. It
35 is also a major target of the host immune system, inducing
neutralizing antibodies, protective immunity, as well as
antibodies which inhibit hemagglutination.

Dengue virus is the causative agent of dengue
fever and is transmitted to man by Aedes mosquitoes,
principally Aedes aegypti and Aedes albopictus. Classic
dengue fever is an acute illness marked by fever, headache,
5   aching muscles and joints, and rash. A fraction of cases,
typically in children, results in more extreme forms of
infection, i.e., dengue hemorrhagic fever (DHF) or dengue
shock syndrome (DSS). Without diagnosis and prompt medical
intervention, the sudden onset and rapid progress of DHF/DSS
10   can be fatal.

Dengue is one of the most important virus groups
transmitted to man by arthropods in terms of global
morbidity; it has been estimated that dengue is responsible
for up to 100 million illnesses annually. With the advent
15   of modern jet travel, dengue has spread globally in the
tropics and subtropics, and multiple dengue serotypes in a
region are common.

Every flavivirus genome is a single
positive-stranded RNA of approximately 10,500 nucleotides
20   containing short 5' and 3' untranslated regions, a single
long open reading frame (ORF), a 5' cap, and a
nonpolyadenylated 3' terminus. The ten gene products
encoded by the single, long open reading frame are contained
in a polyprotein organized in the order, C (capsid), prM/M
25   (membrane), E (envelope), NS1 (nonstructural), NS2a, NS2b,
NS3, NS4a, NS4b, and NS5 (Chambers, T.J. et al. Ann Rev
Microbiol (1990) 44:649-688). Processing of the encoded
polyprotein is initiated cotranslationally, and full
maturation requires both host and viral-specific proteases.
30   The sites of proteolytic cleavage in the YF virus have been
determined by comparing the nucleotide sequence and the
amino terminal sequences of the viral proteins. Subsequent
to initial processing of the polyprotein, prM is converted
to M during virus release (Wengler, G. et al. J Virol (1989)
35   63:2521-2526), and anchored C is processed during virus
maturation (Nowak et al. Virology (1987) 156:127-137).

There are four antigenically related dengue
viruses which, however, can be recognized as distinct
serotypes. The complete genomic sequence for at least one
strain of each of the four dengue serotypes has been
reported (DEN-1, Fu, J. *et al.* Virology (1992) 188:953-958;
DEN-2, Deubel, V. *et al.* Virology (1986) 155:365-377; Hahn,
Y.S. *et al.* Virology (1988) 162:167-180; DEN-3, Osatomi, K.
et al. Virus Genes (1988) 2:99-108; Osatomi, K. *et al.*
Virology (1990) 176:643-647; DEN-4, Zhao, B.E. *et al.*
Virology (1986) 155:77-88; Mackow, E. *et al.* Virology (1987)
159:217-228). In addition, the compete genomic sequences of
other flaviviruses are known (e.g., YF virus: Rice *et al.*,
Science (1985) 229:726-733).

It does not appear that infection by one dengue
serotype can confer long-term immunity on the individual
with respect to other serotypes. In fact, secondary
infections with heterologous serotypes are quite common. In
general, antibody responses in infected subjects to primary
infection are mostly IgM antibodies and these antibodies are
directed against type-specific determinants. On the other
hand, secondary infections by heterologous serotypes
generate IgG antibodies which are flavivirus crossreactive.

Helpful reviews of the nature of the dengue
disease, the history of attempts to develop suitable
vaccines, and structural features of flaviviruses in general
as well as the molecular structural features of the envelope
protein of flaviviruses are found in Halstead, S.B. Science
(1988) 239:476-481; Brandt, W.E. J Infect Disease (1990)
162:577-583; Chambers, T.J. *et al.* Annual Rev Microbiol
(1990) 44:649-688; Mandl, C.W. *et al.* Virology (1989)
63:564-571; and Henchal, E.A. and J.R. Putnak, Clin
Microbiol Rev (1990) 3:376-396.

Other flavivirus family members, i.e., JEV, TBE
and YF appear presently to represent single serotypes --
i.e., antibodies elicited in response to one strain are
cross reactive with other known strains.

A successful vaccine for protection against flavivirus infection has never been developed. However, there have been a number of preliminary efforts, many of which focus on the envelope protein, since this protein is
5 exposed at the surface and is believed to be responsible for eliciting immunity.

Monoclonal antibodies (Mabs) directed against purified E of several flaviviruses, DEN-2 (Henchal *et al*. _Am J Trop Med Hyg_ (1985) _34_:162-169, TBE (Heinz, F.X. *et al*.
10 _Virology_ (1983) _126_:525-537), St. Louis encephalitis (SLE, Mathews, J.H. *et al*. _J Immunol_ (1984) _132_:1533-1537), Murray Valley encephalitis (MVE, Hawkes, R.A. *et al*. _J Gen Virol_ (1988) _69_:1105-1109), and JEV (Takegami, T. *et al*. _Acta Virologica_ (1982) _26_:312-320) are neutralizing in vitro.
15 Some of these Mabs can also passively confer protection in vivo (Heinz, F.X. *et al*. (1983, *supra*)); Mathews, J.H. *et al*. (1984, *supra*)); Kimuro-Kuroda and Yasui, _J Immunol_ (1988) _141_:3603-3610).

Although the primary amino acid sequence of the
20 flavivirus E glycoprotein is variable (45-80% identity), all have twelve conserved cysteine residues, forming six disulfide bridges, and hydrophilicity profiles are nearly superimposable, suggesting that they may all have similar secondary and tertiary structures. Based on the position of
25 the 12 conserved cysteines (determined for West Nile virus, Nowak and Wengler, _Virology_ (1987) _156_:127-137), monoclonal antibody competitive binding studies, monoclonal antibody binding to purified proteolytic fragments, and analysis of neutralizing antibody escape mutants of Tick-Borne
30 Encephalitis Virus, glycoprotein E was divided into three antigenic domains (A, B, and C) and two transmembrane segments at its carboxy-terminus. See, for example, Mandl, C.W. *et al*. _J Virol_ (1989) _63_:564-571. Figure 1, reproduced from this article, shows the locations of these domains.
35 Domain A was defined as a denaturation sensitive, highly folded, and disulfide stabilized discontinuous domain composed of the amino acids from 50-125 and 200-250

containing five of the six disulfide bridges.
Neutralization and hemagglutination inhibition epitopes are
found within domain A, and, for dengue viruses, one of the
two N-linked glycosylation sites. A conserved hydrophobic
5   amino acid sequence within domain A has been postulated to
provide fusogenic properties after low pH treatment. Amino
acid sequences conserved among the flavivirus family are
located within this region; thus, broadly
flavivirus-cross-reactive epitopes lie within this domain.
10   Domain B was identified as a continuous domain
composed of amino acids 301-395 (an approximate region
between amino acids 300-400 for all flaviviruses). The
domain can be isolated as a single immunoreactive
proteolytic fragment. It has been postulated that this
15 domain forms part of the receptor binding site (Heinz, F.X.
et al. APMIS (1993) 101:735-745), and attenuating mutations
have been mapped to sequences within domain B Heinz et al.
(supra). A variety of neutralizing antibodies have been
shown to specifically map to Domain B (Heinz et al. (1983,
20 supra)); Trirawatanapong et al., 1992; Megret et al., 1992;
Lin et al., 1994). The binding of these neutralizing
monoclonal antibodies is dependent on formation of a
disulfide bond, and in some cases also is sensitive to
detergent denaturation. Species-specific monoclonal
25 antibodies bind this domain.
          Domain C represents a hypervariable loop between
the two segments of Domain A. Its antigenicity is
insensitive to denaturation or reducing agents, and contains
one N-linked glycosylation site. Predominantly sub-type
30 specific monoclonal antibodies react with this domain. No
specific activity has been assigned to this domain.
          Many strategies are currently under investigation
to develop an effective and safe dengue vaccine; however, to
date, no single strategy has proven completely satisfactory.
35 Attempts to generate live attenuated dengue vaccine strains
have not been entirely successful, although research into
this area continues. In the absence of effective, live

attenuated dengue vaccines, a significant effort has been
invested in the development of recombinant, dengue subunit
or viral-vectored vaccines.

Recombinant dengue proteins have been expressed in
5   several systems to date (see Putnak, R.A. (1994) Modern
Vaccinology, E. Kurstak ed., Plenum Medical, New York, pp.
231-252, for review). Most efforts using Escherichia coli
have yielded poor immunogen unable to elicit neutralizing
antibodies. This may reflect nonnative conformation of
10   dengue proteins expressed in the bacteria and the necessity
to process the viral proteins through the secretion pathway
in order to form the proper disulfide bonds, glycosylate the
proteins, or both.

Several reports have described vaccinia-flavivirus
15   recombinants expressing envelope protein as part of a
polyprotein (e.g. C-prM-E-NS1; [Dengue] Zhao, B.G. et al. J
Virol (1987) 61:4019-4022; Deubel, V. et al. J Gen Virol
(1988) 69:1921-1929; Bray, M. et al. J Virol (1991)
63:2853-2856; [YF] Hahn, Y.S. et al. Arch Virol (1990)
20   115:251-265), as a single protein (e.g. 100%E; [Dengue]
Bray, M. et al., J Virol (1989) 63:2853-2856), or as
polypeptides (e.g. 79%E-RKG; Men, R. et al. J Virol (1991)
65:1400-1407). The most successful recombinant vaccinia
viruses, those capable of inducing neutralizing antibodies
25   and protecting mice from virus challenge, were those which
were secreted E extracellularly or accumulated E on the cell
surface.

Men, R. et al. (1991, supra) described the
recombinant production of various C-terminal truncations of
30   the DEN-4 envelope protein using a recombinant Vaccinia
virus vector and infecting mammalian CV1 cells. The results
showed that the recombinants that contain greater than 79%
of the coding sequence produced an intracellular protein
that could be immunoprecipitated with anti-dengue virus
35   antibodies contained in hyperimmune mouse ascitic fluid
(HMAF). Although there was a reduced level of detection for
protein which contained 79% of envelope or less, this did

not appear to result from reduced production of the protein.
It was also found that only truncations which contained 79%
of E or less were secreted efficiently; E polypeptides equal
to or larger than 81%E were not secreted efficiently.

5   Men *et al.* (1991, *supra*) constructed additional C-
terminal truncations between 79%E and 81%E to map the amino
acids responsible for the difference in secretion and
immunoreactivity with HMAF of these two truncated E
polypeptides. The results demonstrated that 79% E
10   containing the additional tripeptide sequence RKG was also
secreted. Although both 59% E and 79% E-RKG were secreted,
only 79% E-RKG was detected at the cells' surface. The
recombinant Vaccinia viruses containing various truncations
were also used to immunize mice. Mice immunized with
15   recombinants expressing 79%E-RKG or larger portions of the
envelope protein were protected. However, except for 59% E,
mice immunized with 79% E or a smaller product were only
partially protected. The 59%E elicited high protection
rates (>90%) comparable to 79%E-RKG and larger C-terminal
20   truncated E polypeptides. Protection correlated with
binding to HMAF.

              Combinations of immunogenic structural and
nonstructural JEV virus, DEN-1, DEN-2, and DEN-4 proteins
have been expressed by baculovirus recombinants(Matsuura, Y.
25   *et al.* Virology (1989) 173:674-682; Putnak, R.A. *et al.* Am J
Trop Med Hyg (1991) 45:159-167; Deubel, V. *et al.* Virology
(1991) 180:442-447). Baculovirus-expressed dengue and JE E
glycoprotein elicited neutralizing antibodies, protected
mice from a lethal dengue virus challenge, or both. In
30   spite of these successes, the expression levels reported in
baculovirus are low and the recombinant protein is less
immunogenic than the viral protein (Putnak, R.A. *et al.* Am J
Trop Med Hyg (1991) *supra*).

              Research with purified polypeptides released by
35   proteolysis of flavivirus envelope proteins, with
recombinant polypeptides, and with synthetic peptides has
indicated where protective epitopes may map. The isolated

9000 dalton domain B trypsin fragment from TBE virus
spontaneously refolds and is stabilized by disulfide bridges
(Winkler, G. *et al.* J Gen Virol (1987) 68:2239-2244). This
disulfide stabilized fragment elicited neutralizing
5   antibodies in mice (Heinz, F.X. *et al.* Virology (1984)
130:485-501). In contrast, a 28,000 dalton trypsin fragment
from WN virus containing domain B sequences was unable to
spontaneously refold and did not elicit neutralizing
antibodies (Wengler and Wengler, 1989).
10   A cyanogen bromide-cleaved 8 kD fragment (amino
acids 375-456) overlapping domain B from JEV envelope
protein was found to induce neutralizing antibodies in mice
(Srivastava, A.K. *et al.* Acta Virol (1990) 34:228-238).
Immunization of mice with a larger polypeptide (JE E amino
15   acid 319 to NS1 amino acid 65) spanning the 8 kD peptide
expressed in Escherichia coli as a fusion to protein A
elicited neutralizing antibodies and protected mice from
lethal virus challenge (Srivastava, A.K. *et al.* Microbiol
Immunol (1991) 35:863-870). This polypeptide begins between
20   the two cysteines within domain B, and, therefore, cannot
form the stabilizing disulfide bond that earlier reports
suggest is necessary for presentation of protective
epitopes.
            Immunization of mice with synthetic peptides
25   corresponding to amino acids within domain B, aa 356-376
from MVE (Roehrig, J.T. *et al.* Virology (1989) 171:49-60) or
aa 352-368 from DEN-2 virus (Roehrig, J.T. *et al.* Virology
(1990) 177:668-675), elicited low levels of neutralizing
antibodies in mice, suggesting the presence in domain B of a
30   weak linear neutralizing epitope (Roehrig, J.T. *et al.* 1989
and 1990, *supra*).
            Mason, P.W. *et al.* J Gen Virol (1990) 71:2107-2114
identified two domains of the DEN-1 envelope protein:
domain I which includes amino acids 76-93 of the E protein
35   and domain II (equivalent to domain B) which includes amino
acids 293-402. These domains were identified from deletion
analysis using recombinant fusion proteins expressed in

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E. coli and reacted with antiviral monoclonal antibodies.
Recombinant fusion proteins containing *E. coli* trpE
sequences fused to the envelope protein (amino acids 1 to
412) elicited antibodies in mice which reacted with a
5   portion of the protein containing domain II.

In addition, Mason, P.W. *et al.* (*J Gen Virol*
(1989) 70:2037-2049) expressed a collection of *E. coli trpE*
fusion proteins to segments of JEV virus envelope protein
spanning domain B. The *trpE* fusion proteins containing the
10 smallest JEV E fragments that retained immunoreactivity with
a panel of neutralizing monoclonal antibodies included amino
acid residues from methionine 303 through tryptophan 396.
However, animals immunized with immunoreactive *trpE* fusion
polypeptides did not produce neutralizing antibodies nor
15 were they protected from lethal challenge.

Trirawatanapong, T. *et al.* Gene (1992) 116:139-150
prepared several truncated forms of dengue 2 envelope
proteins in *E. coli* for epitope mapping, and used the
neutralizing monoclonal antibody 3H5 to locate its
20 corresponding epitope. This was first localized between
amino acids 255 and 422. Targeted gene deletions in the
plasmid constructs encoding the truncated proteins permitted
mapping of the binding site to the 12 amino acids between
positions 386 and 397. The mapping was apparently confirmed
25 by the ability of a synthetic peptide containing E protein
amino acids 386-397 to bind 3H5 specifically.

Megret, F. *et al.* Virology (1992) 187:480-491
prepared 16 overlapping fragments of DEN-2 envelope protein
as trpE fusion products in *E. coli* for epitope mapping. The
30 fusion proteins are produced intracellularly and obtained
from the lysates. These products were used to map epitopes
defined by a panel of 20 monoclonal antibodies. Six
antigenic domains were described: nonneutralizing
antibodies bound to peptides containing amino acids 22-58,
35 amino acids 304-332, amino acids 60-97, and amino acids
298-397. Neutralizing antibodies bound to peptides
containing amino acids 60-135, 60-205, and 298-397.

Significantly, Megret *et al.* (1992, *supra*)
demonstrated that all MAbs (including 3H5), with two
exceptions (below), that recognize "full-length" domain B
(amino acids 298-397) are unable to recognize slightly
5   shorter polypeptides. For example, in contrast to the
findings of Trirawatanapong *et al.* Gene (1992, *supra*), MAb
3H5 was unable to bind to trpE fusion proteins containing
DEN-2 E amino acids 304-397, 298-385, or 366-424. The two
exceptional MAbs in the findings of Megret *et al.* are MAbs
10   5A2 and 9D12. The pattern of binding of MAb 5A2 suggests
that it recognizes a linear epitope between amino acids 304
to 332, while MAb 9D12 binds to a polypeptide, amino acids
298-385, which is slightly shorter than the smallest
polypeptide (amino acids 298-397) to which MAb 3H5 binds.
15   These results indicate that both the disulfide bond in
domain B and the domain B C-terminal amino acids are
involved in forming the immunodominant domain B epitopes.
     U.S. Patent No. 5,494,671, issued 27 February 1996
discloses C-terminally truncated dengue and JEV envelope
20   proteins for use in vaccines. This patent is the U.S.
counterpart of PCT Application WO 92/03161 published 5 March
1992. The truncated E proteins were either generated *in
situ* using viral vectors, or in insect cells, also using
viral vectors.
25   Although it appears established from the art that
the B domain and subunits representing at least about 80% E
(truncated at the C-terminus), of the flavivirus envelope
protein contain epitopes which bind neutralizing antibodies,
problems have arisen with respect to producing recombinant
30   polypeptides, all of which contain the B domain, in a form
which mimics the native protein and is thus capable of
eliciting an immune response. The only recombinantly
produced E polypeptides containing the B domain that
elicited a protective immune response in mice were expressed
35   from Vaccinia and baculovirus vectors. Generally,
recombinantly produced proteins lack the appropriate

glycosylation, folding, and disulfide bond formation for
producing a proper immune response.

It has now been found that the B domain and other
shortened forms of the envelope protein can be successfully
5   secreted from yeast and from *Drosophila* in a form which
elicits the production of neutralizing antibodies. This
permits, for the first time, the production of a successful
recombinantly produced subunit flavivirus vaccine.

10   Disclosure of the Invention

The invention provides vaccines containing, as an
active ingredient, a secreted recombinantly produced the
flavivirus envelope protein or a subunit thereof. The
vaccines are capable of eliciting the production of
15   neutralizing antibodies against the relevant flavivirus. In
the illustrations below, the B domain of the envelope
protein (E) is secreted from yeast by producing it in an
expression vector containing the α-mating factor
prepropeptide leader sequence (preproMFα$_L$). Peptide
20   subunits representing 60%E and 80%E are secreted from
Drosophila cells using the human tissue plasminogen
activator secretion signal sequence for the propeptide
(tPA$_L$) or from the homologous premembrane (prM) leader.
100%E can also be so produced. The secreted products can
25   easily be purified and prepared as a vaccine.

Thus, in one aspect, the invention is directed to
a vaccine for protection of a subject against infection by
flavivirus. The vaccine contains, as active ingredient, the
envelope protein of a flavivirus serotype or a subunit
30   thereof. The E or subunit is secreted as a recombinantly
produced protein from eucaryotic cells under the control of
a nonviral promoter. The vaccine preferably contains
portions of more than one flavivirus serotype E protein
similarly produced, and may further include additional
35   flavivirus proteins, particularly NS-1, which can be
similarly recombinantly produced.

'In other aspects, the invention is directed to
methods to protect subjects against infection by
administering the invention vaccines, to antibodies
generated in a mammalian subject administered an immunogenic
5   amount of the vaccine; immortalized B cell lines which
generate monoclonal antibodies of this type; methods to
effect passive immunization by administering the antibodies
of the invention; methods to detect the presence or absence
of antiflavivirus immunoglobulins utilizing the secreted
10   recombinantly produced peptides of the invention and the
recombinant materials important in, methods for, their
production.

Brief Description of the Drawings

15   Figure 1 is a drawing reproduced from Mandl, *et
al.* (*supra*) showing a model of the envelope protein of
flaviviruses.
             Figure 2 shows the partial nucleotide sequence for
DEN-2 PR159 S1 mutant strain and differences from the wild-
20   type strain reported by Hahn (1988, *supra*).
             Figure 3 shows the partial nucleotide sequence and
deduced amino acid sequence of the genome of DEN-2 PR159/S1
strain in comparison with wild-type.
             Figure 4 shows the oligonucleotide used to
25   mutagenize an 80%E cDNA clone to obtain the domain B coding
sequence.
             Figure 5 shows the construction of a cloning
vector containing the nucleotide sequence encoding domain B.
             Figure 6 shows the insertion of the domain B
30   coding sequence into the yeast expression vector pLS5.
             Figure 7 shows the preproMFα$_L$/domain B fusion
protein.
             Figure 8 shows the construction of an expression
vector for domain B in *Pichia*.
35   Figure 9 shows the nucleotide and deduced amino
acid sequence for the tPA$_L$-DomB fusion protein.

Figure 10 shows the survival times of mice
immunized with recombinant domain B and challenged with
Dengue-2.

Figure 11 shows the construction of an expression
5   vector for production of domain B in *Drosophila melanogaster*
tissue cultured Schneider cells.

Figure 12 shows the standard curve for a DomB
sandwich assay.

Figure 13 shows the survival times of mice
10 immunized with *D. melanogaster* Schneider cell-secreted 80%E.

Modes of Carrying Out the Invention

The invention provides, for the first time, a
subunit vaccine that can be efficiently produced
15 recombinantly and secreted that is effective in protecting
subjects against infection with flavivirus. Although many
attempts have been made to obtain such a subunit vaccine,
either the subunit itself is resistant to recombinant
production techniques which permit it to be secreted in a
20 processed form so as to render it effective as an immunogen,
or, if its recombinant production is facile, it fails to
elicit neutralizing antibodies. The present applicants have
found that certain portions of the envelope protein of
dengue virus type 2, such as domain B representing
25 approximately 100 amino acids of the envelope protein
extending approximately from the Gly at position 296 to the
Gly at position 395, and optionally including additional E
sequence through position 413 of the protein, and other
portions of E, i.e., 60%E and 80%E as well as 100%E are
30 effectively secreted by certain convenient eucaryotic
recombinant hosts, in a form that permits processing to
mimic the native conformation of the protein. The hosts
generally utilize nonviral control sequences. The secretion
of the protein into the culture medium facilitates
35 purification. Furthermore, this form is able, when
administered, especially in the presence of adjuvant, to
raise neutralizing antibodies in animals. Thus, this

subunit represents a useful component of a vaccine for
protecting subjects against dengue infection.

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As used herein, "B domain" refers to a peptide
which spans from approximately Gly 296 to Gly 395 of the
DEN-2 envelope protein, and optionally including additional
E sequence through position 413 of the envelope protein.
The corresponding positions in other flaviviruses define the
B domain generally. These positions are approximate; for
example, Mandl (1989, supra) describes the generation of a
tryptic fragment containing domain B which spans the amino
acids of the TBE E protein from position 301 to 396. The
sequences illustrated in the present application represent
the envelope protein from dengue Type 2 virus (DEN-2); three
additional distinct dengue serotypes have been recognized.
Therefore, "Domain B" also refers to the corresponding
peptide region of the envelope protein of these serotypes,
of other flaviviruses and to any naturally occurring
variants. In addition, B domain includes extended forms of
the about 100-120 amino acid peptides, wherein the
extensions do not interfere with the immunogenic
effectiveness or secretion of the B domain. In one
embodiment, such extensions are minimal -- i.e., not more
than six additional amino acids -- at either the N-terminus
or the C-terminus, or distributed between these termini;
preferably no more than four total additional amino acids,
and most preferably no more than two.

The form of domain B which spans positions of
about 296-395 is referred to herein as "classical" domain B.
When the B domain includes at least portions of the region
extending to amino acid 413, the additional region may
confer additional functions, e.g., enhancing immunogenicity
by providing a helper T cell epitope. The form of domain B
which includes positions about 296-413 (of DEN2) and
corresponding positions in other flaviviruses, is referred
to herein as DomB+T. The domain B of the invention includes
these two specific embodiments, "classical" domain B and
DomB+T, as well as those forms which span positions

approximately 296 to a position between position 395 and
413.

Other portions of the E protein illustrated below
are self-explanatory. 80%E is the N-terminal 80% of the
protein from residue 1 to approximately residue 395. 60%E
represents the corresponding shorter sequence. These
precentages are approximate and may include 1-4 amino acid
residues more or less than that calculated precisely. These
subunits are produced from vectors containing the DNA
encoding the mature subunit or protein or may be included in
a prM fusion which results in secretion of the 80% or 60%E
per se.

For practical large-scale production of the
subunits used as active ingredients in the vaccines of the
invention, recombinant techniques provide the most practical
approach. However, to be useful as active ingredients, the
subunits as produced must assume a conformation and undergo
processing under conditions which render them similar to the
native envelope portion as it exists in the envelope protein
of the virus. In order to achieve this, the recombinant
production must be conducted in eucaryotic cells, preferably
yeast or *Drosophila* cells. In addition to the *S. cerevisiae*
and *P. pastoris* yeasts illustrated below, other yeasts, such
as *Kluveromyces sp Yarrowia lipolytica*, and
Schizosaccharomyces pombe may be used. Other insect cells
besides the *Drosophila melanogaster Schneider* cells
illustrated below may also be employed. Additional
appropriate eucaryotic cells include mammalian expression
cells such as Chinese hamster ovary cells. The B domain or
60%E or 80%E must be produced as a correctly processed
protein and secreted.

It has been found, as demonstrated hereinbelow,
that particularly efficient secretion of biologically active
mature protein can be achieved in several ways. First, this
can be done by expressing the protein in yeast in operable
linkage with the α-mating factor signal sequence.
Constructs which place the nucleotide sequence encoding the

E protein or subunit disposed so as to encode a fusion
protein with an upstream α-mating factor signal sequence are
therefore included within the scope of the invention.   An
additional preferred embodiment employs *Drosophila* cells and
5   the human tissue plasminogen activator leader sequence for
secretion of 60%E or 80%E as well as domain B, and other E
proteins and subunits.   This general expression system is
disclosed in EP 290261 published 11 September 1988.
Envelope protein subunits that represent N-terminal portions
10 of truncated protein may also be secreted from the
homologous prM fusion.   Other secretion signal peptides or
secretion leader pre/pro peptides, such as those associated
with invertase or acid phosphatase of *S. cerevisiae* or with
glucoamylase of *C. albicans* or of *A. niger* or the bovine
15 chymosin prepropeptide secretion leader can also be used.
Secretion leaders in general that occur at the amino
terminus of secreted proteins and function to direct the
protein into the cellular secretion pathway generically can
be used.   In general, the invention includes expression
20 systems that are operable in eucaryotic cells and which
result in the formation of envelope protein or a subunit
secreted into the medium.   Preferably, nonviral control
sequences are employed.   Thus, useful in the invention are
cells and cell cultures which contain expression systems
25 resulting in the production and secretion of the E protein
or subunit.

        The properly processed E protein or subunit is
recovered from the cell culture medium, purified, and
formulated into vaccines.   Purification and vaccine
30 formulation employ standard techniques and are matters of
routine optimization; however, particularly advantageous and
novel purification methods are described below.   Suitable
formulations are found, for example, in <u>Remington's</u>
<u>Pharmaceutical Sciences</u>, latest edition, Mack Publishing
35 Company, Easton, PA.   In particular, formulations will
include an adjuvant, such as alum or other effective

adjuvant.   Alternatively, the active ingredient and the
adjuvant may be coadministered in separate formulations.

          The active vaccines of the invention can be used
alone or in combination with other active vaccines such as
5   those containing attenuated forms of the virus.   The
vaccines may contain only one subunit as an active
ingredient, or additional isolated active components may be
added.   Corresponding or different subunits from one or
several serotypes or flavivirus types may be included in a
10   particular formulation.

          More specifically, the subunits utilized in the
vaccines of the invention can include the E protein or
subunits thereof from each or a subset of the four dengue
serotypes, and/or E protein or subunits from alternative
15   flavivirus such as Japanese encephalitis virus, tick-borne
encephalitis virus and the yellow fever virus.   Multiple
subunits of the E protein can also be included, as well as,
optionally, other viral proteins, particularly the NS1
protein.   These alternative subunits derived from other
20   proteins of the virus can be recombinantly produced in a
manner similar to the E protein and subunits described
above.

          Thus, typical combinations might include:

          80%E of the four dengue serotypes plus 60%E of
25   JEV;

          80%E of JEV;

          80%E of TBE plus 60%E of dengue;

          80%E of the four dengue serotypes plus NS1 protein
of four dengue serotypes;

30   80%E of two dengue seroptypes plus NS1 protein or
fragment from JEV;

          Domain B of JEV plus 80%E of TBE;

          Domain B of YF plus 80%E of JEV plus 80%E of
dengue type 2;

35   60%E of JEV plus domain B of four dengue
seroptypes plus NS1 or fragment of both dengue and JEV;

          80%E of JEV plus NS1 of JEV;

80%E of TBE plus NS1 of TBE

80%E of YF plus NS1 of YF.

To immunize subjects against dengue fever or other
flavivirus infections, the vaccines are administered to the
5   subject in conventional immunization protocols involving,
usually, multiple administrations of the vaccine.
Administration is typically by injection, typically
intramuscular or subcutaneous injection; however, other
systemic modes of administration may also be employed.
10   Although the technology is not as well developed,
transmucosal and transdermal formulations are included
within the scope of the invention as are effective means of
oral administration. The efficacy of these formulations is
a function of the development of formulation technology
15   rather than the contribution of the present invention.

Since the subunit vaccines elicit the production
of neutralizing antibodies, the antibodies thus raised can
themselves be used as passive vaccines. For production of
passive vaccine, a suitable mammalian subject is immunized
20   with the E protein or subunit of the invention and
antibodies are either recovered directly as a composition
from the antisera or indirectly as monoclonal antibodies
from immortalized B cells of the subject. For production of
monoclonal antibodies, the conventional techniques of Kohler
25   & Milstein, for example, or treatment with Epstein Barr
virus, are used in immortalizing peripheral blood
lymphocytes or spleen cells and screening for antibodies
immunoreactive with the immunogen. These antibodies may
further be screened for their ability to effect plaque
30   reduction in infected sera or cultures.

The polyclonal antisera are generally subjected to
purification techniques such as standard size separation and
chromatographic techniques to obtain purified
immunoglobulins. The recombinantly produced proteins of the
35   invention are particularly useful affinity ligands for
chromatographic purification. A multiplicity of techniques
is available for the purification of immunoglobulins for use

in passive vaccines. If monoclonal antibodies are to be
purified from small volumes of a medium ascites fluid, a
protein A affinity column is particularly useful. For
larger volumes, additional standard chemical techniques,
5   such as ammonium sulfate precipitation and DEAE
chromatography can be used. These immunoglobulins or the
monoclonal antibodies generated by the immortalized B cells
are then used in vaccine formulations as is understood in
the art.

10   As is the case with active vaccines, the passive
vaccines of the invention may be used in combination with
additional antibodies or tandem administration of the
antibodies of the invention and additional antibodies may be
employed.

15   In the event that the passive vaccine is intended
for use in a species other than that of the subject in which
the antibodies were prepared, it may be desirable to modify
the antibodies to minimize any immunogenic response. For
example, it may be possible to use only the variable regions
20   of these antibodies, such as the F_{ab}, $F_{ab'}$, or $F_{(ab')_2}$

regions. These fragments can be prepared either from
polyclonal antisera or from the supernatants of hybridoma
cultures by treating with proteolytic enzymes and recovering
the desired fragments. The fragments are readily separated
25   by using the relevant protein of the invention as an
affinity reagent.

            Alternatively, chimeric antibodies can be produced
wherein the constant region corresponding to the species to
be protected is substituted for the constant region
30   characteristic of the species of antibody origin. The
availability of recombinant techniques makes the production
of chimeric antibodies a relatively trivial exercise.
Briefly, a hybridoma or cell line producing the antibody of
interest is used as a source for the genes encoding the
35   antibody. The genes are recovered from, for example, the
hybridoma using standard cloning procedures. The genes are
then manipulated *in vitro* to remove the constant region and

replace it with a constant region of a different species
origin. The modified genes are then ligated into expression
systems and expressed in recombinant host cells, such as CHO
cells, monkey cells, yeast cells, and the like.

5   Further modifications in the variable regions can
also reduce immunogenicity. Again, since recovery of the
genes encoding the antibody is within the skill of the art,
the variable regions, too, can be manipulated to replace the
framework regions with framework regions more representative
10   of the desired species, leaving intact the complementarily
determining regions responsible for antigen specificity. In
still another embodiment, the variable heavy chain and
variable light chain regions can be linked through a peptide
linker and produced as a single chain F_V molecule.

15   Thus, if the passive vaccines are intended for
humans, the foregoing various techniques of humanizing
antibodies can be employed to minimize any immunogenic
response even though the original antibodies are raised in
nonhuman species.

20   In addition to use in vaccines or in the
generation of passive vaccines, the mature recombinant E
protein and subunits of the invention may be used as
analytical reagents in assessing the presence or absence of
antiflavivirus antibodies in samples. The interest in doing
25   this may be diagnosis of infection with flavivirus,
monitoring response to infection or may simply reside in the
use of immunoassays as part of standard laboratory
procedures in the study of the progress of antibody
formation or in epitope mapping and the like. The antigen
30   is employed in standard immunoassay formats with standard
detection systems such as enzyme-based, fluorescence-based,
or isotope-based detection systems. Preferably, the antigen
is used coupled to solid support or in sandwich assays, but
a multiplicity of protocols is possible and standard in the
35   art.

          Thus, the secreted protein, such as 60%E, 80%E or
B domain may be adsorbed onto solid support and the support

then treated with a sample to be tested for the presence of
antiflavivirus antibodies. Unbound sample is removed, and
any bound antibodies are detected using standard detection
systems, for example, by treating the support with an

5   antispecies antibody with respect to the species represented
in the sample to be tested, the antispecies antibody coupled
to a detection reagent, for example, horseradish peroxidase
(HRP). The presence of the HRP conjugated antispecies
antibody is then detected by supplying a suitable

10 chromogenic substrate.

     Alternatively, the anti-subunit or antiflavivirus
antibody may be adsorbed to the solid support and detected
by treating the solid support with the recombinant domain B,
either directly labeled, or labeled with an additional

15 antibody in a sandwich-type assay.

     In addition, both the mature peptides, such as
domain B and 60%E or 80%E of the invention and the
antibodies immunoreactive with it can be used in standard
purification procedures as affinity reagents. Thus,

20 purification of the subunits from recombinantly produced
cultures can be effected by affinity columns using
antibodies raised against these antigens. Conversely,
immunoglobulins useful in passive vaccines can be readily
purified from antisera using the peptides of the invention.

25   The mature domain B or other subunit of the
invention may be used to detect the presence or absence of
antibodies of various isotypes, including IgG and IgM
isotypes. As set forth above, detection of IgM isotypes is
significant since this is an index of primary infection.

30   In the examples below, particular subunits of the
dengue Type 2 envelope protein, in particular 60%E, 80%E and
domain B are illustrated as representative of effective
subunits of the envelope protein in flavivirus in general.
For the 60%E and 80%E constructs in general, secretion can

35 be obtained from constructions designed to express the prME
subunit fusion or under control of the human tissue
plasminogen activator leader sequence. The mature

N-terminus of the envelope protein is then secreted into the
culture medium. Whether the N-terminus of the envelope
protein subunits were fused to a heterologous leader, such
as the human tissue plasminogen activator leader sequence,
5   or to the homologous prM sequence or C-terminal portion
thereof, the mature form of the truncated envelope protein
is secreted. The secreted truncated Es are expressed at
high levels in Drosophila, efficiently processed, and
secreted into the medium. The products are glycosylated and
10   processed to an endo-H resistant form. The secreted form of
truncated E produced cotranslationally with prM generally
represents about 20-30% of the total protein in the medium.
Furthermore, based upon reactivity with conformationally
sensitive monoclonal antibodies, using a ELISA and
15   immunofluorescence formats, the secreted E products are
shown to have a native conformation. Immunization of mice
with crude medium from transformed cells expressing prM-
truncated E induces a potent virus-neutralizing response.
        The following examples are intended to illustrate
20   but not to limit the invention.

                        Example 1
              Preparation of Envelope Proteins
                  in Saccharomyces cerevisiae
25   A cDNA clone derived from dengue serotype 2
(DEN-2) described by Hahn, Y.S. et al. Virology (1988,
supra) was used as the starting material. This cDNA derives
from strain PR159/S1. This strain has a small plaque,
temperature-sensitive phenotype; the complete sequence of
30   the cDNA derived from the genomic RNA for PR159/S1 is set
forth in this publication.
        Figure 2 shows the sequence of the cDNA derived
from genomic RNA of DEN-2 PR159/S1 for the Capsid,
preMembrane, Envelope, and NS1 genes. Shown in bold at
35   nucleotides 103, 1940, 1991, and 2025 are corrections to the
Hahn published sequence. Differences in the S1 sequence
from the wild-type sequence are noted above the wild-type

sequence. There are no nucleotide differences in the Capsid and preMembrane protein-encoding portions and there are four in the E encoding portion.

Figure 3 shows the cDNA sequence of DEN-2 PR159/S1
5   for the Capsid, preMembrane, Envelope, and NS1 genes and the inferred translation of those four genes, which is part of the larger dengue polyprotein. The four differences between wild-type DEN-2 PR159 and the S1 strain are shown above the S1 nucleotide sequence. Also shown is the context of the
10 codons in which the nucleotide differences occur and the encoded amino acid.

In the E gene, three of the four mutations are silent; S1 has G instead of A at position 1314, T rather than G at position 1356, and C rather than T at position
15 2025. The mutation at position 1858, a G in S1 rather than A, results in a coding change from Ile in wild-type to Val in S1. There is, therefore, a one amino acid difference in the B domain in the S1 strain as compared to wild-type. Although the amino acid substitution conferred by the
20 mutation at 1858 is conservative, mutagenesis studies of other viral structural proteins (Coller, B.G. *et al.* (1994) <u>Mutagenesis Studies on the Predicted VP2 E-F Loop of Coxsackievirus B3</u>, Abstract, 13th Annual Meeting of the American Society for Virology) have demonstrated that even
25 relatively conservative single amino acid changes in surface loops can have profound effects on viral biology, including infectivity and viral stability. Thus, it is not without foundation that the conservative mutation in domain B of DEN-2 PR159/S1 may be involved in the attenuation of this
30 small-plaque, temperature-sensitive variant.

Subsequent determinations on the S1 strain used herein (S1/HBG) showed that the A at position 103 in wild-type was retained in position 103 in S1/HBG and C was substituted for T at position 2025 (a silent mutation). A
35 deduced amino acid sequence from DEN-2 PR159/S1 and differences occurring in the wild-type are shown in Figure 3.

Various E gene subclones were obtained which
represented the amino-terminal 90% of the envelope, 80% of
the envelope, 60% of the envelope and classical domain B.
Using the assignment of Mandl, C.W. *et al.* J_Virol (1989)
5   63:564-571, classical domain B starts at Gly296 and ends at
Gly395. Classical domain B contains only one disulfide bond
and is encoded by the sequence following domains A and C.
This classical domain B does not contain the potential T
cell epitope described by Mandl *et al.* at its carboxy end
10   which can be included in some forms of the domain B of the
invention, e.g., DomB+T.

The portion of the genome that encodes 80% of the
envelope protein (80%E) was amplified using the Polymerase
Chain Reaction, primers D2E937p and D2E2121m, and plasmid
15   pC8 (Hahn *et al.* (1988, *supra*) as template.

In this notation of the primers, the virus
serotype is first indicated (D2 for DEN-2), then the
corresponding dengue gene -- i.e., in this case envelope, E,
is noted. Then is noted the number in the dengue cloned
20   sequences of Figures 2 or 3 for the first dengue nucleotide
in the 5'-3' direction of the oligonucleotide, i.e., using
the numbering of Hahn *et al.* (1988, *supra*), and finally the
notation shows whether the oligonucleotide primes the plus
(p) or the minus (m) strand synthesis. The sequence in the
25   primers corresponding to dengue cDNA are written in
uppercase letters; nondengue sequence is written in
lowercase letters.

D2E937p2
30

         *Bgl*II
     5'-ct<u>tctaga</u>t<u>ctcgag</u>ta<u>cccggg</u>acc ATG CGC TGC ATA GGA ATA TC-3'
         *Xba*I   *Xho*I   *Sma*I

35

D2E2121m

         *Sal*I
     5'-gc<u>tctaga</u>gtcga cta tta TCC TTT CTT GAA CCA G-3'
40   *Xba*I   End End

The D2E2121m primer placed two stop codons after
the 395th codon of E. The 80%E amplified cDNA fragment was
digested at the *Xba*I sites in the cloning adapters and
cloned into the *Nhe*I site of pBR322 to obtain p29D280E.

5   Double-strand sequence for 80%E was determined, which
identified a single silent PCR-introduced mutation at
nucleotide 2001 (AAC/Asn to AAT/Asn).

A subclone representing domain B was obtained from
the 80%E subclone by oligonucleotide-directed mutagenesis.

10   In the mutagenesis, stop codons and restriction endonuclease
sites were inserted between domain C- and domain B-encoding
sequences. The stop codons were positioned to terminate
domain A+C translation and SalI and PvuII restriction sites
were added to facilitate subcloning of domains A+C and

15   domain B fragments, respectively, into yeast expression
vectors. As shown in Figure 4, to avoid a high AT content
in the mutagenic oligonucleotide, the stop codons defining
the carboxy-terminus of 60%E containing domains A and C were
positioned four codons upstream of the beginning of domain

20   B, i.e., following Lys291. The original and altered
nucleotide sequences of the mutagenized region and the
corresponding amino acid translation are shown in Figure 4.

To perform the mutagenesis, a 580 bp BamHI
fragment spanning domain B from the pBR322-80%E clone

25   p29D280E was subcloned into pGEM3Zf (Promega) to yield
p29GEB2. (See Figure 5.) This BamHI fragment encodes the
3' end of domains A and C and all of domain B. The plasmid
obtained following mutagenesis, p29GEB24PS, was confirmed by
DNA sequencing. As shown in Figure 6, the introduced PvuII

30   site permits direct subcloning of domain B cDNA into yeast
expression vectors as a fusion with yeast secretion leader.

The cloned cDNA fragments encoding B domain and
80%E were inserted into expression vectors so as to maintain
the translational frame of fusions to secretion leaders as

35   described below. The sequence of the fusions were confirmed
by DNA sequencing.

Example 2
Secretion of Domain B from *Saccharomyces cerevisiae*

The expression vector constructed to secrete classical domain B from *Saccharomyces cerevisiae* to include envelope protein amino acids 296-395 was constructed so that processing by the proteases normally involved in preproMFα$_L$ processing would yield a precisely processed domain B. One of these proteases, Kex2p, the gene product of the *Kex2* gene, cleaves following dibasic peptides, such as LysArg, LysLys, ArgArg, and ArgLys. A second protease involved, a dipeptidyl amino peptidase, removes GluAla and AspAla dipeptides from the amino terminus after Kex2p processing.

The MFα prepropeptide (MFα$_L$)-domain B fusion was operably linked to the constitutive promoter from the gene (*TDH3*) encoding glyceraldehyde phosphate dehydrogenase (GAPDH) contained in pLS5 and, alternatively, to the copper sulfate-inducible *CUP1* gene promoter contained in pLS6. These vectors were provided by SmithKline Beecham. Both contain the MFα$_L$ sequence, and StuI, BglII, and SalI cloning sites, and use the *TRP1* gene as a selectable marker. They contain sequences derived from pBR322 to provide an *E. coli* origin of replication, the ampicillin resistance gene, and sequences derived from the 2-micron plasmid of *S. cerevisiae* to enable replication in *S. cerevisiae*. The insertion of the classical domain B coding sequence into the appropriate reading frame in pLS5 was accomplished by digesting pLS5 with StuI and SalI and by digesting p29GEB24PS with PvuII and SalI and gel purifying the small PvuII-SalI domain B fragment and ligating it into the thus opened pLS5 as shown in Figure 6. A domain B expression vector derived from pLS6 was constructed in a similar manner.

The nucleotide and amino acid sequence of the MFα$_L$-domain B fusion is shown in Figure 7.

The resulting expression vectors were transfected into spheroplasts of *Saccharomyces cerevisiae* strain GL43 (*MATatrp1Δ1 ura3-52 proA::URA3*) and transformants were

selected by their ability to grow on minimal medium without
tryptophan supplementation. *S. cerevisiae* strain GL43 was
supplied by SmithKline Beecham. Strain GL43 pLS5-DomB and
pLS6-DomB transformants were grown in small (<5ml) cultures
5   i Casamino acid-supplemented minimal medium, and proteins
secreted into the culture medium and cellular proteins were
analyzed by Coomassie-stained SDS-PAGE and Western blot. A
novel Coomassie-staining band of approximately 12 kD, which
was weakly immunoreactive with DEN-2 HMAF, was observed in
10 the culture media of domain B transformants. No novel
Coomassie-staining bands and little or no immunoreactive
protein not found in negative controls was observed in
cellular protein extracts of domain B transformants. When
cultured in Casamino acid supplemented minimal medium,
15 comparable levels of domain B were secreted into the medium
by pLS5-DomB and pLS6-DomB transformants. About 20% of the
total secreted protein was domain B as estimated by the
intensity of Coomassie brilliant blue staining of protein in
an SDS-PAGE polyacrylamide gel.

20   The composition of the medium was optimized; when
S. cerevisiae containing pLS5-DomB were grown in minimal
medium (SD) or Casamino acids (Difco)-supplemented minimal
medium, the yields of protein were less than when cultured
in rich (YEPD) medium. The pLS5-domain B transformant was
25 preferred since this transformant can be cultured in rich
medium whereas pLS6-domain B transformants require the use
of the less preferred supplemented minimal medium in order
to induce with copper sulfate.

            The secreted protein was confirmed as domain B
30 having a Glu-Ala dipeptide appendage to the N-terminus,
designated herein Glu-Ala-Domain B or Glu-Ala-DomB. For
this demonstration, proteins secreted by a pLS5-DomB
transformant were separated by SDS-PAGE and electroblotted
to Immobilon P membrane (Millipore), and the amino terminal
35 amino acid sequence was determined by microsequencing. That
sequence is: H_2N-Glu Ala Gly Met Ser Tyr Ser Met Xxx Thr
Gly Lys Phe Xxx Val Val. The persistence of the GluAla

dipeptide indicates that the dipeptidyl aminopeptidase
incompletely processed the domain B N-terminus following
proteolysis by Kex2p.

5   Example 3
                     Purification of Domain B
A.   Initial Purification Method:
          Pilot purifications of domain B from *S. cerevisiae*
culture medium used size exclusion chromatography on
10   acrylamide Biogel P100.
          For these purifications, an *S. cerevisiae* GL43
pLS5-DomB transformant was cultured in minimal medium
overnight and then transferred to either Casamino acid-
supplemented minimal (SD) medium or rich (YEPD) medium for
15   expression. After culturing at 30°C for 2-3 days and feeding
daily with buffered glucose to final concentrations of 0.4%
glucose and 5 mM sodium phosphate (pH 6.7), the cells were
pelleted by centrifugation and the medium was clarified by
filtration through a 0.45 μm pore filter. The filtered
20   medium was concentrated about 30-fold either by tangential
flow or by centrifugal ultrafiltration using Minitan
(Millipore) or Centricon (Amicon) devices, and the
concentrate was exchanged into PBS azide buffer (2.7 mM KCl,
1.2 mM KH_2PO_4, 138 mM NaCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 0.02% azide)
25   by diafiltration or dialysis.
          In one example, the concentrated secreted proteins
from approximately 500 ml of culture of GL43 pLS5-DomB grown
in SD plus Casamino acids were loaded onto a 2.5 x 75 cm
Biogel P100 column and domain B was eluted from the column
30   at approximately 1 bed volume. Domain B was pooled based on
SDS-PAGE analysis of column fractions. The pooled domain B
fractions were brownish in color and could not be
decolorized by dialysis. The brown colored preparation also
was not immunoreactive in ELISAs. DEAE chromatography of
35   this sample resulted in binding of the brown color and a
mostly colorless flow-through containing domain B. For DEAE

chromatography, the brown-colored pooled fractions were
dialyzed against 0.1 M acetic acid, pH 5.1 and loaded onto a
1.4 x 15 cm DEAE (Biorad) column. Domain B was eluted using
a 0.01 M acetic acid, pH 5.1, 0.1 M NaCl step gradient.

5   The purified domain B was of high purity as
assessed by silver stain on SDS-PAGE. The resulting Glu-
Ala-DomB also gained weak reactivity with antidengue mouse
polyclonal HMAF and with antidengue MAbs 3H5 and 9D12 in an
ELISA format. The reactivities of earlier colorless
10   preparations from smaller cultures were greater than this
initial large-scale preparation. For the ELISA, 96-well
microtiter plates were coated with domain B by overnight
incubation at 4°C and then blocked with BSA in 50 mM tris-
HCl, pH 7.0, 0.15 M NaCl, 0.05% Tween for 1 hour at room
15   temperature. The plates were then treated with either DEN-2
HMAF or monoclonal antibody. Bound antibody was detected
with alkaline phosphatase conjugated goat antimouse IgG and
measured by the increase in optical density at 414 nm using
the alkaline phosphatase substrate paranitrophenylphosphate.
20   The domain B frequently eluted from the Biogel
P100 in two equal fractions, the first with the bulk of the
secreted yeast proteins and the second as 70-90% pure domain
B. Treatment with 1M NaCl, 1 and 2 M urea, and 1% DDAPS, a
zwitterionic detergent, were ineffective in completely
25   disaggregating domain B during size exclusion
chromatography. Since the domain B of both fractions had
the same electrophoretic mobility in the presence of
detergent and absence of DTT, this polypeptide was neither
covalently cross-linked to another protein nor self-
30   polymerized via disulfide bonds.

B.   Improved Purification Method:
          A standard culture and purification method was
developed that has reproducibly yielded immunoreactive
35   domain B expressed and secreted by *S. cerevisiae* GL43 pLS5-
DomB. For that method, a primary culture is prepared by
inoculating 60 ml of SD medium in a 250 ml baffled flask

with a single colony of yeast strain GL43 pLS5-DomB grown on
an SD plate. SD medium is prepared by autoclaving 0.67%
Bacto yeast nitrogen base without amino acids, cooling and
providing 2.0% w/v dextrose and 12 mM K_2HPO_4 using autoclave
5   stock solutions. Solid SD medium is prepared by including
Bacto agar at 1.8%. The culture is grown at 30°C for 18-24
hours with shaking at 240 rpm.

Secondary cultures are then grown in 300 ml of SD
medium supplemented with 0.2% casamino acids in 2 L baffled
10   flasks. The secondary cultures are inoculated with 30 ml of
the primary culture. The secondary cultures are incubated
at 30°C for 18-24 hours with shaking at 300 rpm.

Large-scale tertiary cultures are then prepared by
inoculating 1 L of YEPD medium in a 4 L baffled flask with
15   100 ml of the secondary culture. YEPD is prepared by
autoclaving 1% yeast extract plus 2% Bactopeptone, to which
sterile dextrose is added to a final concentration of 2%
after cooling. The large-scale cultures are incubated at
30°C for 48 hours with shaking at 300 rpm. After 24 hours,
20   the culture is supplemented with 0.01 vol of sterile 40% w/v
glucose and 0.02 vol of sterile 1 M phosphate buffer,
pH 6.7.

After growth in the 1 L culture, the cells are
removed by centrifugation at 5,000 rpm at 4°C for 5 min. in a
25   Sorvall GS-3 rotor. EDTA and EGTA are added to final
concentrations of 1 mM each to the cleared medium, and the
resulting solution is filter sterilized using a 045 μm pore
filter membrane (Millipack-20 or Opticap-50, Millipore).
Glycerol is added to 10% v/v to the filtrate which is then
30   concentrated 20-30 fold using tangential flow
ultrafiltration (Millipore Minitan System) with two membrane
cartridges (four regenerated cellulose membranes) of a 10 kD
MW cutoff. The retentate is kept on ice during
ultrafiltration in the coldroom at 4°C for about 10-15 hours.

35   The concentrated medium is directly used or
rapidly frozen on dry ice/ethanol in 10 ml aliquots in 15 ml

polypropylene or polystyrene tubes with a screw cap and
stored at -70°C.

Concentrated medium of the tertiary culture is
dialyzed at 4°C using a membrane with a 6-8 kD cutoff
5   (Spectra/Pore-1 Membrane) against 10 mM acetate, pH 4.5 (4X4
liters; 2-3 hours each; 1 overnight).

For purification, the concentrated, buffer-
exchanged medium is loaded onto a 2.5 x 22 cm CM Biogel A
(Biorad) column at 0.8 ml/min. at 4°C. The column is washed
10   with 150-250 ml of 10 mM acetate, pH 4.5 until the optical
density at 280 nm of the eluent drops to baseline. Domain B
is then eluted with 10 mM acetate, 300 mM NaCl, pH 4.5 at
0.8 ml/min. Fractions of approximately 8 ml are collected
and analyzed by SDS-PAGE (15%) and visualized by silver
15   staining. Domain B migrates on the trailing edge of the
main peak of eluted material as monitored by 280 nm.

The domain B-containing fractions are pooled and
concentrated about 11-fold by centrifugal ultrafiltration
using, for example, a Centriprep-10 (Amicon) and loaded onto
20   a 5 x 60 cm Sephadex G-75 (Superfine, Pharmacia) column at
4°C. The column is eluted using phosphate-buffered saline
(PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4, 0.24 g/l
KH_2PO_4) at a flow rate of 0.8 ml/min. Nine ml fractions are
collected and analyzed by SDS-PAGE as above and by indirect
25   ELISA as above using Mab 9D12.

The Sephadex G-75 fractions containing pure
immunoreactive domain B from 2-4 purification runs are
concentrated about 50-fold by centrifugal ultrafiltration
using a Centriprep-10 (Amicon; final volume 2-5 ml). The
30   pooled material is stored at 4°C.

Glu-Ala-DomB purified as above typically is
immunoreactive with the virus-neutralizing monoclonal
antibodies 3H5 and 9D12. The above-described culturing and
purification protocol has been performed several times with
35   reproducible results. The number of cultures of the above-
indicated volumes can be increased, and we have grown

simultaneously up to six 1 L tertiary cultures. The culture
medium from the multiple cultures is pooled for
clarification and concentration, and domain B from the
concentrate is purified in batches and pooled following
5   verification of immunoreactivity. The amount of protein in
the purified domain B preparations may be quantitated by
total amino acid analysis using HCl hydrolysis with on-line
ninhydrin reaction and optical detection. Table X presents
the yield of protein in purified domain B from four
10   cultures.

Table X			
Total Protein Yields in Purified Domain B Preparations			
Lot No.	Culture Volume	Final Yield*	Yield mg/l
198.76	850 ml	4 mg	4.7
198.71	1500 ml	35 mg	23
244.68	5750 ml	90 mg	16
244.190	5000 ml	25 mg	5

Based on amino acid composition of total protein.

15   Example 4
Production of Domain B in *Pichia pastoris*
        In addition to *Saccharomyces cerevisiae* as used in
the previous examples, the methylotrophic budding yeast *P.
pastoris* was used as a host. The domain B coding sequence
20   was subcloned into the expression vector pPIC9 (Invitrogen,
San Diego, CA) which contains the alcohol oxidase (AOX1)
promoter sequence from *P. pastoris*, the MFα prepropeptide
secretion leader from *S. cerevisiae*, the AOX1
transcriptional termination sequence, and the *HIS4* gene from
25   *S. cerevisiae* as a selectable marker. To construct pPIC9-
DomB, the domain B-encoding XhoI-SalI fragment from pLS5DomB
was inserted into XhoI-digested pPIC9 as shown in Figure 8.
XhoI cuts at identical positions within the MFα$_L$-encoding
DNA of both pLS5 and pPIC9, and *SalI* cuts immediately
30   following the translational stop codons following domain B-
encoding sequences. After verification by DNA sequence

analysis, the expression cassette, consisting of the *AOX1*
promoter-MFα$_L$-domain B-*AOX1* terminator, plus HIS4 was
released from pPIC9-DomB by BglII digestion and the linear
DNA was transformed into competent *P. pastoris* cells

5 according to the supplier's instructions (Invitrogen, San
Diego, CA). Transformants were selected for the ability to
grow on medium lacking histidine (His$^\pm$ phenotype), and
specific integration of the transforming DNA at the AOX1
locus was indicated in some transformants by slow growth on

10 medium containing methanol (Mut$^\underline{s}$ phenotype) as the sole
carbon source.

The expression and secretion of domain B from
eleven *P. pastoris* pPIC9-DomB transformants in 3 ml cultures
was surveyed by Coomassie-stained SDS-PAGE analysis. A

15 protein of molecular weight equal to domain B secreted by
S. cerevisiae was evident in the culture media of ten of the
eleven *P. pastoris* transformants. Immunoblots of a
comparable polyacrylamide gel demonstrated that the protein
secreted by *P. pastoris* pPIC9-DomB transformants was

20 immunoreactive with polyclonal antibodies made to domain B
secreted by *S. cerevisiae* (see Example 6). The SDS-PAGE
analysis indicated that *P. pastoris* secreted domain B at a
level comparable to or higher than that achieved in
S. cerevisiae.

25 For further comparison of the secreted expression
levels of domain B by *S. cerevisiae* and *P. pastoris*, the
S. cerevisiae transformant and the two best *P. pastoris*
transformants, Nos. 5-8 (Mut$^\pm$) and 6-16 (Mut$^\underline{s}$), were cultured
in 100 ml shake flask cultures. *S. cerevisiae* pLS5-DomB was

30 cultured 48 hrs in YEPD medium, and 100 ml precultures of
the *P. pastoris* pPIC9-DomB transformants were grown in BMGY
medium (1.34% yeast nitrogen base without amino acids, 2%
Bacto peptone, 0.4 μg/ml biotin, 1% glycerol, and 100 mM
potassium phosphate, pH 6.8) for 24 hrs and then harvested

35 and resuspended in 25 ml of BMMY heterologous protein-
inducing medium (the same as BMGY, except with 0.5% methanol
replacing the glycerol) and cultured 48 hrs. Culture media

with cells removed by sequential centrifugation and
filtration through a 0.45 μm pore size membrane were buffer
exchanged by diafiltration into TEEN (10 mM Tris, pH 8.0, 1
mM EDTA, 1 mM EGTA, and 150 mM NaCl). The relative levels
5   of domain B secreted by the three cultures were estimated by
size fractionating the proteins in 3, 6, 9, and 12 μl of
each sample on a 15% SDS-PAGE gel and staining with
Coomassie blue. As estimated by the intensity of Coomassie
staining, *P. pastoris* secreted at least four-fold more
10   domain B per volume of induction culture medium than
S. cerevisiae in YEPD. Correcting for the four-fold
concentration of culture volume of the *P. pastoris* when
transferred into the induction medium, we conclude that
P. pastoris secretes slightly more than *S. cerevisiae* in
15   shake flask cultures on a per volume basis. However, on a
per gram cell dry weight basis, *P. pastoris* (0.80 gm total
cell dry weight) secreted approximately 1.6-fold more domain
B than *S. cerevisiae* (1.33 gm total cell dry weight).

20   Example 5
                    Effect of Extending Domain B
          In a manner similar to that set forth in the
preceding examples, two plasmid constructs for expression in
S. cerevisiae were made that include additional domain B-
25   carboxy terminal sequence downstream of amino acid 395 with
the domain B coding sequence. One construct, designated
DomB+stem, represents Gly296-Gly445 and was expressed as a
fusion with the MFα$_L$. This construct includes those
sequences that lie between domain B and the transmembrane
30   anchor of E. This region contains a potential T cell
epitope (Mandl *et al.* J Virol (1989) 63:564-571) and
additional hydrophobic sequences, a peptide from this region
elicits virus-recognizing antibodies (Roehrig *et al.* 1922 in
Vaccines 92, pp. 2777-2281), and this region may contribute
35   to the proper folding and presentation of the domain B B-
cell epitope to the immune system.

The domain B+stem cDNA fragment was constructed in
E. coli cloning vectors by combining the domain B cDNA
fragment and the 3' end of a 90%E clone. As introduced in
Example 1, an E gene subclone representing the amino

5   terminal 90% of E was constructed from DEN-2 PR159/S1 cDNA
plasmid pC8 of Hahn *et al.* (1988, *supra*) using the PCR. The
90%E polypeptide contains all of E except for the C-terminal
membrane anchor comprising two transmembrane domains. The
90%E cDNA clone was made as follows. The 90%E fragment was

10   amplified by the PCR using pC8 as template and primers
D2E937p and D2E2271m. The sequence of D2E937p is given in
Example 1. The sequence of D2E2271m is:

D2E2271m:
15   *SalI*
         5'-gctctagagtcga cta tta CCC GTA GAT TGC TCC G-3'
             XbaI   End End

20   Primer D2E2271m placed two stop codons after Gly445 of #,
and the two primers positioned useful restriction enzyme
sites at both ends of the fragment. The PCR-amplified 90%E
cDNA fragment was made blunt at both ends and cloned into
the *SmaI* site of a modified pUC13 cloning vector, yielding

25   pVZ90E.
         Combining domain B and the 90%E 3' end made use of
a unique *AflIII* restriction enzyme site found in most pUC-
like cloning vectors and a unique *AflIII* site in domain B
sequences. This combining was accomplished by first

30   subcloning the 90%E fragment from pVZ90E into pBluescript to
reverse the orientation of 90%E relative to the vector
sequences, yielding pBS90E. Then, p29GEB24PS, containing
domain B sequences in pGEM (Example 1), and pBS90E were
digested with *AflIII*, and the vector-domain-B5' fragment and

35   the domain-B3'-stem-vector fragment from the two digestions,
respectively, were purified, ligated, and recovered in
E. coli yielding pBS-Bstem.
         For expression in *S. cerevisiae*, the *PvuII-SalI*
domain B+stem fragment from pBS-Bstem was subcloned into the

pLS5 yeast expression vector at its StuI and SalI sites, putting the MFα_L and B+stem in translational frame. The sequence of the ligated junctions were verified by DNA sequence determination. The domain B+stem expression

5   vector, pLS5-BStem, was transformed into S. cerevisiae strain GL43. The resulting transformants grew more slowly than did transformants with pLS5-DomB containing classical DomB and the plasmid appeared to be rapidly lost during growth under nonselective conditions. The DomB+stem product

10   may be toxic to the cells, and the hydrophobicity of the stem may inhibit secretion.

      However, a similar construct representing Gly296-Ala413 analogously inserted into pLS5 resulted in substantial amounts of secreted domain B protein. This form

15   of domain B, designated herein DomB+T, contains the downstream T cell epitope. As stated above, the DomB proteins of the present invention include classical DomB and all or part of the amino acid sequence downstream from Gly395 to the Ala residue at position 413.

20   The DomB+T cDNA fragment was amplified by the PCR from pC8 plasmid DNA using oligonucleotide primers D2E1822p and D2E2175m. The primer sequences are:

D2E1822p KpnI XhoI

25   5'-cta gcg gta ccc tcg aga AAA GGG AGG CCG GGA TGT CAT ACT CCA TGT GC-3'

D2E2175m SalI NotI

30   5'-cgt gtg tcg acg cgg ccg cta tta GGC CAT TCT TTT CGC TCC-3'

The PCR product was cloned into pUC18 following KpnI and

35   SalI digestion of the product. The DomB+T cDNA fragment was then released from pUC18-DomB+T by XhoI-SalI digestion and cloned into the XhoI site in pLS5 within the MFα_L and Gly395 of domain B+T. S. cerevisiae strain GL43 secrets approximately ten-fold more domain B than domain B+T.

40

Example 6
Preparation of Antibodies to the
Recombinant Domain B Protein

The Glu-Ala-DomB peptide which had been column-
purified, denatured by heating in SDS, and reduced by
treatment with DTT was used to immunize Swiss Webster mice
following removal of the excess SDS. The mice yielded high-
titre antibodies that were highly immunoreactive when used
to probe Western blots displaying the same antigen or DEN-2
viral envelope protein.

Example 7
Recombinantly Produced Glu-Ala-Domain B
in ELISA Assays

The serological diagnosis for dengue infection is
based on the detection of anti-dengue IgM and IgG in primary
and secondary viral infection using standard Enzyme Linked
Immunosorbent Assay (ELISA) techniques. Current assays are
based on the ability of anti-dengue immunoglobulins to
recognize semi-purified virus. Primary and secondary
infections can be distinguished by the IgM:IgG ratios.
(Innis et al., 1989; Kuno et al., 1991).

The recombinantly produced Glu-Ala-domain B
purified as in paragraph B of Example 3 was tested as an
antigen preparation in both IgM and IgG tests. In these
ELISAs, the antigen was coated on plates, followed by sera
positive for dengue antibodies and then detection by goat
antihuman antibody.

Microtiter plates were coated overnight at 4° C
with 100 μl of a 5 μg/ml solution of domain B. After
blocking with 3% normal goat serum, primary infected dengue
2 positive serum at a 1:100 dilution was added per well.
The bound IgM was detected by the addition of goat anti-
human IgM (Fc) conjugated to horseradish peroxidase. Under
these conditions, the recombinant Glu-Ala-domain B did not
provide positive results.

For IgG, high titer secondary dengue 2 infected
sera were supplied at a 1:270 dilution and detected with
goat anti-human IgG (Fc) conjugated to horseradish
peroxidase. Two of the five sera gave positive reactions in
5 this assay.

The recombinant Glu-Ala-domain B purified by the
improved procedure of Example 3 is not recognized by murine
polyclonal hyperimmune ascitic fluid (HMAF) to other dengue
serotypes and to other flaviviruses, when assayed using a
10 similar ELISA format. Flavivirus infected murine sera
tested include, Japanese Encephalitis virus, Tick-Borne
Encephalitis virus, Yellow Fever virus, Saint Louis
Encephalitis virus, West Nile virus, three viral isolates of
dengue serotype 1, two viral isolates of dengue serotype 3,
15 and two viral isolates of dengue serotype 4.

Sandwich assay for the detection of any domain B-containing
envelope antigen.

In an alternative enzyme immunoassay format, anti-
20 domain B or anti-Dengue capture antibody, polyclonal or
monoclonal, may be absorbed to the solid support, and sample
containing an unknown quantity or serotype of dengue antigen
may be added and then detected by reacting with a second
anti-domain B or anti-Dengue antibody, either conjugated to
25 a signal-generating enzyme or to be detected using a
appropriate signal generating system, of which there are a
multitude. This immunoassay is useful for the quantitation
of recombinantly produced envelope protein or whole virus by
comparing the immunoreactivity of a known concentration of
30 domain B with that of the unknown antigen preparation. In
addition, the conformational sensitivity or epitope binding
site of the anti-domain B capture antibody can be varied to
garner additional information regarding the conformation of
dengue protein in the preparation.

35 To perform the sandwich enzyme immunoassay, 100 μl
of anti-Dengue monoclonal antibody 9D12 or 3H5 (Henchal,
E.A. et al., Am J Trop Med Hyg (1985) 34:162-169) was used

to coat microtiter wells. The monoclonal antibodies were
purified by Protein-A affinity chromatography and used at 10
µg/ml concentration (diluted in PBS: 50 mM sodium Phosphate,
pH 7.0, 0.15 M NaCl). After a one hour incubation, the
5   wells were washed three times with TBS-T (50 mM Tris-HCl pH
7.0, 0.15 M NaCl, 0.05% Tween-20), and blocked with 200
µl/well of 1% BSA in PBS for 1 hour at room temperature.
Following three washes with TBS-T, 100 µl per well of a
standard purified solution of domain B or an unknown sample
10   was added to each well and captured by the bound monoclonal
antibody. The domain B solution was allowed to bind the
antibody for one hour at room temperature, and after three
washes with TBS-T, 100 µl of Protein A affinity purified
polyclonal rabbit anti-domain B serum (25 µg/Ml) diluted in
15   PBS-T (PBS, 0.25% BSA, 0.05% Tween-20) was added to each
well and allowed to bind to the bound antigen. Following
three washes with TBS-T, 100 µl of alkaline phosphatase-
labeled goat anti-rabbit IgG+IgM (H and L) conjugate
(Caltag) diluted 1:2500 in PBS-T (previously established by
20   titration) was added to each well. After incubation for one
hour at room temperature, the plates were washed four times
with TBS-T and 200 µl/well of 1 mg/ml p-nitrophenylphosphate
(pNPP) substrate in alkaline phosphatase substrate buffer
(25 mM Trizma base, pH 9.5, 0.15 M NaCl, 5 mM $MgCl_2$; 0.02%
25   NaN_2) was added. The plates were incubated for one hour at
room temperature and the absorbance of each well was
measured on a Dynatech MR5000 using a sample wavelength of
410 nm and a reference wavelength of 630 nm. A typical
domain B standard curve is presented in Figure 12.
30

Example 8
Immunization of Mice to Raise Neutralizing Antibodies
A.   Crude DomB Immunogenicity in Mice: Media from
yeast cultures secreting DomB and a negative control yeast
35   culture were buffer-exchanged with PBS, and total secreted
proteins were concentrated for injection into mice. Six

groups of five mice each (Outbred Swiss strain, Simonsen) were inoculated with the crude DomB preparation, negative control secreted protein, or saline, as listed below.

Group	Immunogen	Adjuvant
I	Saline	Freund's
II	Saline	none
III	control medium	Freund's
IV	control medium	none
V	DomB medium	Freund's
VI	DomB medium	none

5

Primary immunizations consisted of 50 µg of the immunogen diluted in phosphate buffered saline, with or without complete Freund's adjuvant as indicated above. Three boosts
10 of 25 µg each were delivered at two-week intervals with no adjuvant (Groups II, IV, and VI) or Freund's incomplete adjuvant (Groups I, III, and V). Test bleeds were obtained one week after the first and second boosts, and the mice were bled out one week after the third boost.
15   Western blot and ELISA analysis of the first test bleeds confirmed a strong immune response to DomB among the Group VI (DomB, no adjuvant) mice. After the second bleed, this response was found to be titratable to greater than 1:6,400 by ELISA. Group VI was the only group that
20 developed high titer DomB antibodies. The mice immunized with DomB in Freund's adjuvant (Group V) mounted a weak immune response; based on reactivity to the immunogen in Western blot format and DomB-ELISA antibody titers in the 100-400 range, Group V mice appeared to be immunosuppressed.
25 The control mice (Groups I-IV) were all negative for antibody to DomB in both the Western and ELISA screens. In spite of high DomB antibody titers in Group VI mice, plaque reduction neutralization tests (PRNT) revealed that none of the mice immunized with the crude DomB produced neutralizing
30 antibody (Table 1).

		Table 1					
		Plaque Reduction Neutralization Tests of Sera from Mice					
		Immunized with DomB in Concentrated, Total Secreted Yeast Proteins					
Group	Trial	Dilution Plaque Counts					
PBS + Freund's Adjuvants		1:100	1:200	1:400	1:4	1:8	1:16
	1	109, 105	~100	~100			
	2				34, 33	38, 30	44, 44
PBS		1:10	1:20	1:40	1:4	1:8	1:16
	1	100, 106	~100	~100			
	2				29, 33	33, 40	39, 37
Negative secreted proteins + Freund's		1:10	1:20	1:40	1:4	1:8	1:16
	1	106, 95	~100	~100			
	2				40, 35	37, 39	39, 47
Negative secreted proteins		1:10	1:20	1:40	1:4	1:8	1:16
	1	109, 94	~100	~100			
	2				38, 42	44, 40	45, 42
Crude DomB + Freund's		1:10	1:20	1:40	1:4	1:8	1:16
	1	100, 92	~100	~100			
	2				$37 \pm 5^{*}$	$35 \pm 6^{*}$	$39 \pm 3^{*}$
Crude DomB		1:10	1:20	1:40	1:4	1:8	1:16
	1	105, 91	~100	~100			
	2				$39 \pm 3^{*}$	$41 \pm 3^{*}$	$39 \pm 3^{*}$
DEN-2 HMAF		1:100	1:200	1:400	1:800	1:1600	1:3200
	1	0, 0	0, 0	0, 0	11, 8	31, 19	81, 75
					1:250	1:1000	1:4000
	2				3, 6	12, 15	21, 21

PRNT assays performed on VERO cells (Trial 1) or BHK-21 C15 cells
(Trial 2) with DEN-2 NGC strain. All plaque counts indicate the
number of plaques obtained with the sera from five animals were
pooled and assayed in duplicate, <u>except</u> those indicated (*) where
each serum sample was individually assayed in duplicate and the
number of plaques averaged (mean ± SD).

B.   <u>Pure DomB Immunogenicity in Mice</u>:   In contrast to mice immunized with crude DomB, outbred ICR mice (Charles River) immunized with purified DomB demonstrated high titer DEN-2 virus neutralizing antibodies. Purified DomB, at a

5   concentration of 3.5 mg/ml, was used to immunize mice (three per group) with 175 µg of purified DomB mixed 1:1 with Freund's adjuvant, with alum, or without adjuvant (PBS). Test bleeds taken after three inoculations were assayed by PRNT (Table 2).

10

Table 2 Plaque Reduction Neutralization Tests of Sera from Mice Immunized with Purified DomB								
Group	Trial	Dilution						80% PRNT Titer[1]
		Plaque Counts						
Negative Ascites Fluid		1:10	1:20	1:40				
	1	36,40	35,34	40,38				
	2	39,38	47,45	41,44				
DEN-2 HMAF		1:100	1:200	1:400	1:800	1:1600	1:3200	
	1	0,0	0,0	1,0	2,1	8,9	13,14	1500
	2	0,1	1,0	1,2	1,1	7,6	16,17	>1600
DomB no adjuvant		1:10	1:20	1:40	1:80	1:160	1:320	
	1	36,29	28,36	29,27	36,30	40,25	28,31	>10
	2	9,17	20,15	22,26	33,26	41,36	42,47	~10
DomB alum		1:10	1:20	1:40	1:80	1:160	1:30	
	1	8,6	10,9	10,12	21,20	32,26	30,28	10
	2	1,0	5,4	8,7	12,15	20,25	27,35	40
DomB Freund's		1:40	1:80	1:160	1:320	1:640	1:1200	
	1	0,0	1,0	1,4	9,2	ND	ND	>320
	2	0,0	3,4	5,5	5,20	13,9	23,20	~640

PRNT assays performed on VERO cells with DEN-2 NGC strain. ND = Not Determined.

Mice immunized with purified DomB in the absence of
adjuvant lacked neutralizing antibodies. DomB
administered in combination with alum elicited low titer
(80T PRNT ~1:10) neutralizing antibodies, and mice

5 receiving purified DomB in combination with Freund's
adjuvants had a high PRNT titer (80T PRNT >1:320).
          Three groups of 3 mice each, 5-6 week-old
outbred ICR strain (Charles River) were used.
Inoculation was intramuscularly in the rump at one site

10 using 10 µg of antigen in 0.1 ml administered solution.
Three inoculations were given to each group on days 1,
20, and 43. In one group, inoculation on day 1
incorporated complete Freund's adjuvants, on day 20
incomplete Freund's adjuvant, and that at day 43, no

15 adjuvant. In a separate group, no adjuvant was supplied
and in a third group, alum was supplied with all three
inoculations. The sera were withdrawn on day 57 and the
sera from each group were pooled, heat-inactivated at 56°C
for 30 minutes and tested for their ability to reduce

20 plaques formed from VERO cells.

          C.   DomB Protection from Virus Challenge: Suckling
mice were immunized with purified DomB in Freund's, Alum,
Hunter's TiterMax (Vaxcel), or no adjuvant for protection

25 against an intracerebral injection of DEN-2 New Guinea C
(NGC) strain. DomB administered in all adjuvants
conferred comparable moderate survival against dengue
virus challenges although survival was statistically
significant (P< 0.5 G test) only for mice immunized with

30 DomB and Hunter's TiterMax. The results are shown in
Figure 10.

          D.   KLH-DomB Immunogenicity in Mice: Two series of
mouse immunizations were initiated to determine the 50%

35 effective immunizing dose (EID_{50}) of unconjugated and KLH-
conjugated DomB. Effects of alum and Freund's adjuvants
to a no-adjuvant control are compared.

DomB was conjugated to KLH via EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl) using the carbodiimide coupling method at a 1.5:1 DomB-to-KLH mass ratio. The amount of unconjugated and conjugated DomB used for these immunizations was normalized, relative to the amount of unconjugated DomB. This normalization was based upon the specific immunoreactivity of each preparation as assayed by indirect ELISA.

Mice were immunized with the priming does of 174, 52, or 5.2 µg (total protein) of the KLH-conjugated DomB in Freund's, alum, or no adjuvant. Additional mice were immunized with 87, 26, or 2.6 µg (total protein) of unconjugated DomB in Freund's adjuvant to allow direct comparison of conjugated and unconjugated material. Boosts consisting of one-half the priming dose are being given at two-week intervals. Test bleeds are assayed for the induction of anti-DomB antibodies by ELISA and Western blot. Final bleeds are tested for induction of a neutralizing immune response by PRNT assay as well as for the production of binding antibodies by ELISA and Western blot. The results are summarized in Table 3.

The response to unconjugated DomB with Freund's adjuvants was low compared to the results in Table 2, which had shown that unconjugated DomB induced a strong virus neutralizing response in outbred ICR mice when administered with Freund's adjuvants. This apparent difference may be grounded in testing pooled sera for the data in Table 2. Pooling sera may mask individual variability. The variability in Table 3 may be attributed to the limited epitopes in DomB and to differences in the MHC genes for the outbred Swiss mice used in Table 3.

	Table 3 Mouse Immune Response to Unconjugated and KLH-Conjugated Purified Domain B				
mouse	antigen	adjuvant	Final Titer Western[c]	Final Titer ELISA[c]	Final Titer PRNT$_{50}$[c]
1	saline	none	N/T[a]	<1:100	N/T
2			N/T	<1:100	<1:10
3			N/T	<1:100	<1:10
4	87 μg B	none	N/T	<1:100	<1:10
5			N/T	<1:100	N/T
6			N/T	<1:100	<1:10
7	87 μg B	Freund's	>1:100,000 Dblt[b]	>1:102,400	1:40
8			N/T	<1:100	<1:10
9			1:1,000,000 Dblt	>1:409,600	<1:10
10			N/T	<1:100	<1:10
11			1:100,000 Dblt	1:25,600	<1:10
12	26 μg B	Freund's	N/T	<100	<1:10
13			1:100,000 Dblt	1:102,400	<1:10
14			1:10,000 Dblt	1:102,400	N/T
15			1:10,000 Dblt	>1:25,600	<1:20[c]
16			1:10,000 Dblt	1:409,600	<1:10
17	2.6 μg B	Freund's	1:1,000 Dblt	1:25,600	<1:10
18			N/T	<1:100	<1:46
19			N/T	<1:100	<1:48
20			N/T	<1:100	<1:24
21			N/T	<1:100	<1:10
22	"87 μg" KLH-B	none	N/T	1:100	<1:10
23			<1:100	1:400	<1:24
24			N/T	<1:100	<1:24
25			N/T	<1:100	<1:24
26			N/T	<1:100	<1:20
27	"26 μg" KLH-B	none	N/T	<1:100	<1:34
28			N/T	<1:100	<1:70
29			N/T	<1:100	<1:34
30			N/T	<1:100	N/T
31			N/T	<1:100	<1:10

mouse	antigen	adjuvant	Final Titer Western[c]	Final Titer ELISA[c]	Final Titer PRNT[c]
			Table 3 (Cont'd) Mouse Immune Response to Unconjugated and KLH-Conjugated Purified Domain B		
32	"2.6 Tg" KLH-B	none	N/T	<1:100	N/T
33			N/T	<1:100	<1:10
34			N/T	<1:100	<1:10
35			N/T	<1:100	<1:10
36			N/T	<1:100	<1:10
37	"87 Tg" KLH-B	Freund's	N/T	>1:1,600	1:20
38			N/T	>1:100	<1:10
39			N/T	<1:100	<1:10
40			1:1,000	>1:1,600	1:20
41			N/T	<1:100	<1:10
42	"26 Tg" KLH-B	Freund's	1:10,000 Dblt	>1:25,600	1:10
43			1:10,000 Dblt	>1:25,600	1:2560
44			1:10,000 Dblt	>1:25,600	1:5120
45			N/T	<1:100	<1:10
46			N/T	1:100	<1:10
47	"2.6 Tg" KLH-B	Freund's	N/T	<1:100	<1:10
48			N/T	<1:100	<1:10
49			<1:50	1:6,400	<1:10
50			N/T	<1:100	<1:10
51			<1:50	1:25,600	<1:10
52	"87 Tg" KLH-B	Alum	<1:100	<1:400	1:60
53			N/T	<1:100	<1:10
54			<1:100	>1:1,600	1:320
55			<1:100	>1:1,600	1:80
56			1:1000	>1:6,400	1:640
57	"26 Tg" KLH-B	Alum	N/T	>1:100	<1:10
58			N/T	<1:100	<1:10
59			N/T	<1:100	<1:10
60			N/T	<1:100	<1:10
61			N/T	<1:100	<1:10
62	"26 Tg" KLH-B	Alum	<1:100	1:1,600	1:120
63			1:1000	>1:1,600	1:120
64			N/T	<1:100	<1:10
65			1:100	>1:1,600	1:80
66			N/T	<1:100	<1:10

[a]N/T - not tested
[b]Dblt - a doublet at approximately 12 kD
[c]If serum was insufficient for testing at 1:10 dilution then higher initial dilutions were used and are indicated.

E.   <u>DomB Immunizations for Hybridoma Generation</u>:

Six BALB/c mice were immunized with 87 μg of unconjugated
DomB or 174 μg KLH-conjugated DomB in Freund's adjuvant.
Upon demonstration of a strong anti-DomB response, the
5   mice are sacrificed and their spleen cells fused to
hybridoma cells for monoclonal antibody production.

<u>Example 9</u>
<u>Production of Domain B in *Drosophila*</u>

10   The cloning vector p29GEB2.4PS, containing the
domain B-encoding nucleotide sequence was digested with
PvuII and the short fragment ligated into pMttSma, which
is derived from pMttbns by digesting with BglII and
inserting an adapter oligonucleotide containing an Sma
15   site.   The duplex linker adapter inserted has the
sequence pGATCCCGG. pMttsma then contains a unique SmaI
site at the 3' end of sequences encoding the tPA leader.
The parent vector pMttbns SmithKline contains the
Drosophila metalothionein gene (*mtn*), the human tissue
20   plasminogen activator secretion signal (tPA$_L$) and the
SV40 early polyadenylation signal. The resulting
expression vector, pMttDomB is thus obtained as shown in
Figure 11. The sequence of the fusion is shown in Figure
9.

25   The resulting pMttDomB vector was transfected
into Schneider S2 cells which were maintained in
Schneider medium (Life Technologies, Grand Island, NY)
supplemented with 10% heat-inactivated FBS at 25°C. The
cells were plated the day prior to transfection at 5 x
30   10^5 cells in 60 mm tissue culture dishes in a total
volume of 4 ml Schneider medium plus 10% FBS. The
Schneider cells were then cotransfected with pMttDomB and
pCOHygro (a hygromycin B phosphotransferase-based
selection vector obtained from SmithKline Beecham) using
35   standard calcium phosphate transfection. 48 hours after
transfection, the medium was supplemented with hygromycin

at 300 µg/ml; hygromycin-resistant cells grew in 2-3
weeks. Varying levels of the cotransformed plasmids were
used in the transfection procedure ranging from
expression plasmid: PCOHygro, 1:1 to 100:1.

5   Cells cotransfected at ratios of 1:1, 5:1 and
20:1 were induced with 200 µM copper sulfate and the
media and cells were harvested at days 1, 4 and 7.
Western blots showed secretion of domain B into the
medium.

10

Example 10
Production of 60%E and 80%E in *Drosophila*

Using the adapted expression vector of Example
9, containing the *Drosophila* metalothionein gene, the
15   human tissue plasminogen activator signal and the SV-40
early polyadenylation signal, the nucleotide sequences
encoding 80%E, prM 80%E, 60%E and prM 60%E are inserted
and the resulting vectors used to transform Schneider
cells as described in Example 9. The mature truncated
20   forms of the envelope protein are secreted into the
medium, or properly processed, and are conformationally
correct with respect to the corresponding native portions
of the envelope protein.

Furthermore, crude media from these
25   transformants employed in the protocol set forth in
paragraphs A and B of Example 8 produce antibodies which
are neutralizing against dengue virus.

Example 11
Expression of 80%E in *Saccharomyces cerevisiae*

30   An expression vector (pLS6-80%E) was
constructed for secretion of the N-terminal 80% (codons
1-395) of the DEN-2 PR-159 S1 envelope glycoprotein
(80%E) from *S. cerevisiae*. The 80%E DNA sequences were
35   obtained from plasmid p29D280E, described in example 1,
by restriction endonuclease digestion with both *Bgl*II and
*Sal*I. The released fragment was isolated by agarose gel

electrophoresis and subcloned between the *Bgl*II and *Sal*I
sites of pLS6, a yeast expression vector described in
example 2. The resulting recombinant plasmid, pLS6-80%E,
contains truncated E as a translationally in-frame fusion
5   to the leader region of mating-factor α (MFα), a secreted
yeast protein. The MFα leader (MFα$_L$) contains a 19 amino
acid secretion signal peptide followed by a 66 amino acid
propeptide. Cleavage of the signal peptide is expected
to occur concomitantly with translocation across the
10 endoplasmic reticulum membrane. Maturation of MFα
normally involves removal of the propeptide by Kex2p, a
golgi protease, and subsequent trimming of N-terminal
(Glu/Asp)Ala dipeptides by dipeptidyl aminopeptidase
(DPAP). The herein described MFα$_L$-80%E fusion was made
15 such that processing of the MFα$_L$ propeptide and trimming
of a GluAla dipeptide results in 80%E with eight
additional N-terminal amino acids derived from sequences
present in the multiple cloning site of the pLS6 vector
or in the PCR primer-adapter used to synthesize the 80%E
20 cDNA (see below).

```
     ATG .....GCT......GAG GCC TTT AGA TCT CGA GTA CCC GGG ACC ATG ...GAA    TAA
     TAG
     Met-18aa Ala-65aa Glu Ala Phe Arg Ser Arg Val Pro Gly Thr Met1...Gly395End
25   End
                    ▲       ▲        ▲                              80%E
          Signalase Kex2p  DPAP
```

30 Transcription of the gene fusion is driven by the *S.*
cerevisiae copper-inducible copper metallothionein (*CUP1*)
promoter.
        After confirming the DNA sequence of the ligated
junctions of expression vector pLS6-80%E, the recombinant
35 DNA was transformed into *S. cerevisiae* strain GL43 (*MATa*
trp1Δ1 ura3-52 pep4::URA3; SmithKline Beecham) according
to standard protocols (Gutherie & Fink, eds. 1991; Rose
et al, 1990). Transformants were selected by their

ability to grow on minimal medium (SD medium: Guthrie and
Fink, 1991) without tryptophan supplementation.

    In order to test for expression and secretion of
80%E, several transformants were grown as small-scale

5   cultures (5 ml medium in 17x150 mm tubes). Single
colonies were used to inoculate SD medium and the
inoculated cultures were grown to saturation (overnight,
30°C, 220 rpm). $1x10^8$ cells from the overnight culture
were used to inoculate 5 ml of minimal SD medium

10 supplemented with Casamino acids (2 g/l; Difco) and $CuSO_4$
(200 µM). This expression culture was fed with glucose
(4 g/l, final concentration) and sodium phosphate (pH
6.7, 20 mM, final concentration) at 24 hours post
inoculation and was harvested by centrifugation at 48

15 hours of growth (30°C, 220 rpm, in 17x150 mm tubes).
Cell-free spent medium was buffer-exchanged with TEEN+PIC
(50 mM Tris, 10 mM EDTA, 10 mM EGTA, 150 mM NaCl, pH 8.0
with 1 µg/ml each of pepstatin and leupeptin and 1 mM
phenylmethylsulfonylfluoride) and concentrated 250-fold

20 using Centricon-30 (Amicon) ultrafiltration. An extract
of cellular protein was prepared by lysing the yeast
cells with vigorous agitation in the presence of glass
beads (425-600 µm) and TEEN+PIC using a Mini Beadbeater
apparatus (BioSpec Products, Bartlesville, OK). Samples

25 were endoglycosidase H_f digested according to the
manufacturer's (New England Biolabs, Beverly, MA)
protocol prior to SDS-PAGE analysis. Protein gels were
Coomassie-stained directly as well as Western blotted and
immunoprobed using mouse polyclonal serum raised against

30 recombinant domain B (described in example 15). Negative
control yeast carrying the expression vector without a
Dengue gene insert secreted no proteins recognized by the
anti-domain B serum, while the major immunoreactive band
from pLS6-80%E medium had a relative mobility matching

35 that of other recombinant 80%E proteins (see Example 17).
The pLS6-80%E medium also contained a minor

immunoreactive species with an apparent molecular weight
6-8 kD higher than 80%E; this is likely to be unprocessed
MFα propeptide-80%E. The pLS6-80%E cellular protein
extract contained many immunoreactive polypeptides not

5   observed in negative control cells, two of which match
the secreted products discussed above. A Coomassie-
stained protein corresponding to recombinant 80%E could
not be identified in either the pLS6-80%E secreted sample
or the total cellular protein sample. This indicates the

10 relatively low levels of recombinant protein expression
in these preparations.

Example 12
Construction of expression vector pPIC9-80%E and

15 secretion of 80%E by *P. pastoris* expressing MFα_L-80%E

The expression vector constructed to secrete
80%E from *P. pastoris* was engineered to express amino
acids 1-395 of the DEN-2 PR-159 S1 envelope glycoprotein
as a fusion to the MFα$_L$. The DNA sequences encoding 80%E

20 were obtained from the clone p29D280E, described in
example 1, by digestion with the restriction enzymes *Sma*I
and *Sal*I. The isolated fragment was treated with the
Klenow DNA polymerase I fragment enzyme to make the *Sal*I
end blunt. This 80%E fragment was then cloned into the

25 *Pichia* expression vector pPIC9 (Invitrogen, San Diego,
CA) which contains the MFα secretion leader (MFα$_L$)
sequence, *Sna*BI, *Eco*RI, and *Not*I cloning sites, and uses
the *HIS4* gene as a selectable marker. The described 80%E
fragment was ligated with pPIC9 plasmid DNA that was

30 previously digested with the restriction enzyme *Sna*BI.
The orientation and genetic integrity of the resulting
gene fusion expression vector, pPIC9-80%E, was confirmed
by restriction digestion and DNA sequence analysis. The
organization, partial nucleotide and predicted amino acid

35 sequences of the MFα$_L$-80%E fusion gene are shown below:

```
ATG ......... GAG GCC TTTAGATCTCGAGTACCCGGGACC ATG ...GGA      TAA
Met-18aa-65aa Glu Ala PheArgSerArgValProGlyThr Met₁...Gly₃₉₅ END
             ▲   ▲   ▲                                    80%E
      Signalase Kex2p  DPAP
```

The location of the signalase and *Kex2* cleavage sites
which remove the pre and pro portions of the MFα leader
peptide, respectively, are indicated. The dengue
sequences are indicated in bold type. The Met_1 residue
is the N-terminal amino acid of the E glycoprotein and
Gly_{395} is residue 395 from the amino terminal end of the
envelope glycoprotein. The expression of a recombinant
product in *Pichia* from the pPIC9 vector is driven by the
methanol inducible promoter derived from the *Pichia AOX1*
(alcohol oxidase 1) gene.

The pPIC9-80%E expression vector was
transformed into spheroplasts of *P. pastoris* strain
GS115 (*his4*) and transformants were selected for their
ability to grow on minimal medium without histidine
supplementation. Strain GS115 and the protocol used for
transformation were obtained from Invitrogen (San Diego,
CA). Transformants were tested for their ability to
express and secrete 80%E by growing selected clones in
small cultures (5 to 50 ml). The transformants were
first grown to saturation (24 to 36 hrs.) in BMGY medium
(1% yeast extract, 2% peptone, 100 mM potassium
phosphate, pH 6.0, 1.34% yeast nitrogen base without
amino acids, 4×10^{-5}% biotin, 1% glycerol). The cells
were collected by centrifugation and suspended in one
half the original culture volume with BMMY (identical to
BMGY except the glycerol component of BMGY is replaced in
BMMY with 0.5% methanol) medium and cultured for 48 hrs.
Proteins secreted into the culture medium as well as
cellular proteins were treated with endoglycosidase H_f,
(EndoH$_f$, New England Biolabs, Beverly, MA) and separated
by SDS-PAGE. Protein gels were analyzed by both
Coomassie staining and immunoprobing of Western blots.
When nonEndoH treated samples are compared to EndoH

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treated samples on Coomassie stained gels of proteins
prepared from the culture medium, a unique staining band
of approximately 50 kD is present in the EndoH treated
lanes of all the pPIC9-80%E transformants.

5   Immunoprobing with anti-domain B serum (see example 6)
detected a smear ranging from 50 to 90 kD in the nonEndoH
treated samples and a unique band of approximately 50 kD
in the EndoH treated samples on Western blots of proteins
prepared from the culture medium. No bands were detected

10   by the anti-domain B serum in negative control lanes.
Variable amounts of the corresponding immunoreactive band
were detected in EndoH treated samples of cellular
protein samples. The approximately 50 kD 80%E product
produced by the MFα$_L$-80%E construct is consistent with

15   the approximate molecular weight as determined by SDS-
PAGE of other recombinant 80%E proteins (see Example 17).
The amount of secreted 80%E in the culture medium is
about 1% of the total secreted protein as estimated by
the intensity of the Coomassie staining band detected.

20   In one liter cultures, the amount of 80%E secreted into
the culture medium was determined to be 500 ng/ml by use
of a sandwich ELISA method.

                            Example 13
25   Construction of Expression vector pMttbns-80%E and
         secretion of 80%E by _Drosophila melanogaster_ Schneider
                     cells expressing tPA$_L$-80%E
             The expression vector constructed to secrete
80%E from _Drosophila melanogaster_ tissue culture cells
30   included the sequences encoding the DEN-2 PR159/S1
envelope glycoprotein amino acids 1-395. The DNA
sequences for 80%E were obtained from the clone p29D280E
(described in Example 1) by digestion with the
restriction enzymes _Bgl_II and _Sal_I. The released 80%E
35   fragment was cloned into the _Bgl_II plus _Xho_I-digested _D._
melanogaster expression vector pMttΔXho. The expression
vector pMttΔXho is a derivative of the vector pMttbns

(SmithKline Beecham) that contains a *D. melanogaster*
metallothionein gene promoter (*Mtn*), the human tissue
plasminogen activator leader sequence (tPA$_L$), and the
SV40 early polyadenylation signal. A 15 bp *Bam*HI DNA

5   fragment containing a *Xho*I site was deleted from pMttbns
to make pMttΔXho, in which the *Bgl*II and another *Xho*I
restriction endonuclease sites are unique. This
construction resulted in the 80%E fragment being fused to
the tPA$_L$ sequence. During normal maturation of tissue

10 plasminogen activator the 20 amino acid prepeptide region
of the leader sequence is removed by signalase in the
endoplasmic reticulum and the 11 amino acid propeptide
region is enzymatically removed in the Golgi. The
genetic integrity of the gene fusion expression vector,

15 pMtt80%E, was confirmed by restriction digestion and DNA
sequence analysis. The nucleotide and predicted amino
acid sequences of the tPA$_L$-80E fusion gene are shown
below:

20 ATG .... GGA GCC AGA TCT CGA GTA CCC GGG ACC **ATG**   ... **GGA**   **TAA**
    Met- 20 aa - 11 aa -Gly Ala Arg Ser Arg Val Pro Gly Thr **Met$_1$** ... **Gly$_{395}$** **END**
           pre$^\blacktriangle$ pro-tPA$^\blacktriangle$ **80%E**

25 The tPA pre- and propeptide regions are delineated by pre$^{\cdot}$
and pro-tPA$^{\cdot}$, respectively, and the dengue sequences are
indicated in bold type. The Met$_1$ residue is the N-
terminal amino acid of the envelope glycoprotein and
Gly$_{395}$ is residue 395 from the amino terminal end of the

30 envelope glycoprotein.
          The selection plasmid, pCOHygro (SmithKline
Beecham), carries the *E. coli* hygromycin B
phosphotransferase gene under the transcriptional control
of a *D. melanogaster copia* transposable element long

35 terminal repeat and confers resistance to hygromycin B.
Others have demonstrated that Schneider cells that have
been cotransfected with both the pMtt expression vector
and the pCOHygro plasmid and are then selected for

hygromycin B resistance produce stable cell populations
that contain multiple copies of the cotransfected gene of
interest. *Drosophila melanogaster* Schneider cells (ATCC,
Rockville, MD) were cotransfected with the pMtt80%E and
5   pCOHygro plasmids at a ratio of 20:1 using the calcium
phosphate coprecipitation method (Gibco BRL).
Transformants were selected by outgrowth in Schneider
medium (Gibco BRL) supplemented with 10% fetal bovine
serum (Hyclone) and 300 µg/ml hygromycin (Boerhinger
10   Mannheim). Transformants were split to a cell density of
2 X 10^6 cells/ml in serum free Excell medium (JRH
Biosciences) and induced with 200 µM CuSO$_4$. The medium
and cells from induced cells were separately harvested
after 6-7 days of induction. Proteins secreted into the
15   culture medium were separated by SDS-PAGE, and proteins
were analyzed by both Coomassie staining and
immunoprobing of Western blots. The Coomassie blue-
stained SDS-PAGE gels shows that the approximately 50 kD
secreted 80%E product is one of the predominant proteins
20   in the unconcentrated medium, comprising as much as 20%
of the total protein. Immunoblots probed with anti-DEN2
hyperimmune mouse ascites fluid (HMAF; from Robert
Putnak, Walter Reed Army Institute of Research) and
polyclonal anti-domain B antisera (Example 6) revealed a
25   single immunoreactive polypeptide of approximately 50 kD
present in unconcentrated medium. In addition,
immunoblots revealed that the 80%E produced by the tPA$_L$-
80%E construct was slightly larger than that obtained
upon expression of a tPA$_L$-prM80%E construct (described in
30   detail in Example 17). This additional mass may owe to
the nine adapter amino acids at the amino terminus of
80%E (GARSRVPGT-80%E) when expressed from pMtt80%E versus
80%E expressed from pMttprM80%E (Example 17). The tPA
propeptide, if not proteolytically removed, may also
35   contribute to the additional molecular weight of 80%E
expressed from pMtt80%E.

Example 14
Subcloning of dengue prM100%E and prM80%E cDNAs and
mutagenesis of E secretion signal-encoding sequence
(mutSS)

5   A cDNA clone of DEN-2 PR159/S1 designed to
encode the preMembrane, Membrane, and Envelope genes
(prM100%E) was constructed by PCR amplification
essentially as described in Example 1 for the subcloning
of 80%E. This cDNA clone includes nucleotides 439 to

10 2421 of the DEN-2 genome. The dengue cDNA fragment was
generated using synthetic oligonucleotide primers
D2pM439p and D2E2421m (see example 1 for nomenclature)
and plasmid pC8 (Hahn et al, 1988, *supra*) as template.
In addition to DEN-2 specific sequences, the primers

15 contained the identical adapter sequences described in
Example 1, except that a methionine codon (ATG) was
included immediately preceding the first codon of the
preMembrane sequence (phenylalanine). The primers are:

20 D2prM439p
               BglII
         5'-attctagatctcgagtacccgggacc atg TTT CAT CTG ACC ACA CGC-3'
            *Xba*I   *Xho*I   *Sma*I

25 D2E2421m
               SalI
         5'-tctctagagtcga cta tta GGC CTG CAC CAT AAC TCC
            *Xba*I   END END

30
         The PCR-generated prM100%E cDNA fragment was
digested with the restriction endonuclease *Xba*I and
ligated into the *Xba*I site of pBluescript SK$^+$
(Stratagene, San Diego, CA) to obtain the plasmid

35 p29prME13. DNA sequence analysis of the PCR-generated
cDNA clone identified three PCR-introduced nucleotide
differences between pC8 and p29prME13. A T→C transition
at nucleotide 1255 resulted in a silent mutation, while
the A→G transition at nucleotide 1117 resulted in the

40 conservative amino acid substitution of a valine for an

isoleucine. The final transition observed at nucleotide
1750 replaced a methionine, adjacent to a cysteine
involved in a disulfide bond in domain A, with a valine.
The effect of this substitution on the ability of the
5   disulfide to form or its stability is not known.

        To generate a cDNA subclone representing
prM80%E, a 794 bp *BamHI-SalI* fragment from p29prME13
representing the envelope carboxy terminal-encoding
fragment was removed. This fragment was replaced with a
10   431 basepair *BamHI-SalI* fragment from p29D280E (described
in Example 1) that encodes a 20% carboxy end truncation
of the envelope glycoprotein. The *BamHI* site is a
naturally occurring site within the envelope cDNA, and
the *SalI* site is an engineered site that immediately
15   follows the stop codons encoded by the PCR primers. The
resulting truncated cDNA clone, p48BSprM80E, was
confirmed by restriction digestion and DNA sequence
analysis to encode amino acids 1 through 395 of the
envelope glycoprotein following prM.
20   Expression of the prM80%E cDNA in *S. cerevisiae*
(Example 15) demonstrated absence of proteolytic
processing between the prM and 80%E proteins in this
yeast. To improve processing of E from prM,
oligonucleotide-directed mutagenesis was performed to
25   alter the naturally occurring signalase cleavage site
between the prM and E proteins. Based on the algorithm
of Von Heijne (1986, Nucl. Acids Res. 14:4683-4690), the
natural DEN-2 E secretion signal peptide receives a poor
predictive score for its function as a secretion signal.
30   The algorithm of von Heijne is based on N-terminal amino
acid sequences found in secreted and nonsecreted
proteins. Scores for functional secretion signal
peptides range from 3.5 to 17, with a mean of ~10. The
score for the secretion signal peptide of E of DEN-2
35   PR159/S1 is 5.2, near the lower end of the range for
signal peptides. In the mutagenesis, the sequence
encoding the four amino acids immediately preceding and

the amino acid immediately following the signalase
cleavage site were altered to change these five amino
acids. The modified signal sequence has a score of 12.4
based on von Heijne's algorithm. The original and
5   altered nucleotide sequences of the mutagenized region
and the encoded amino acid sequences relative to the
signalase site are:

Original	ATC	GCT	CCT	TCA	ATG	ACA	ATG	CGC	TGC
	Ile	Ala	Pro	Ser	Met	Thr	Met	Arg	Cys
Mutagenized	ATC	GCT	**GGC**	**GCT**	**CAA**	**GCT**	**CAA**	CGC	TGC
	Ile	Ala	**Gly**	**Ala**	**Gln**	**Ala**	**Gln**	Arg	Cys

                                Membrane │ Envelope
                                         ▲

15   Signalase Cleavage
The mutagenized sequence and the resulting amino acid
changes are indicated in bold.

          To perform the mutagenesis, a 1,122 bp *SmaI-*
HindIII fragment spanning the prM-E signalase cleavage
20   site from the p29prME13 cDNA clone was subcloned into
pAlter1 (Promega, Madison, WI) to yield the plasmid
pAltSmaH3prME. The 1,122 bp *SmaI-HindIII* fragment
contains all of prM and 611 bp of the E sequence. The
HindIII site is a naturally occurring site within the E
25   sequence that is located at nucleotide 1547 of the
genomic sequence. The mutagenized clone,
pAltSmaH3prME(mutSS), was verified by DNA sequence
analysis.

30   Example 15
      Construction of expression vectors pLS6-prM80%E and
       pLS6-prM(mutSS)80%E, expression of MFα$_L$-prM80%E and
        MFα$_L$-prM(mutSS)80%E in *Saccharomyces cerevisiae*, and
      secretion of 80%E by *Saccharomyces cerevisiae* expressing
35   MFα$_L$-prM(mutSS)80%E
          For expression of DEN-2 PR159/S1 preMembrane
protein amino acids 1-166 and Envelope glycoprotein amino

acids 1-395 as a single continuous open reading frame in
S. cerevisiae, DNA sequences encoding these proteins were
obtained by digestion of plasmid p48BSprM80E (see Example
14) with restriction endonucleases *Bgl*II and *Sal*I. This
5   fragment was cloned into the pLS6 yeast expression vector
(see Example 2) that had been digested with *Bgl*II and
*Sal*I. The structure of the resulting gene fusion
expression vector, pLS6-prM80%E, was confirmed by
restriction digestion and DNA sequence analysis. The
10 nucleotide and predicted amino acid sequences of the
MFα$_L$-prM80%E fusion gene are shown below:

                          *Bgl*II   *Sma*II
      ATG .... GAG GCC TTTAGATCTCGAGTACCCGGGACCATG **TTT ...ACA   ATG ...GAA   TAA**
15 Met-18aa-65aa Glu Ala PheArgSerArgValProGlyThrMet **Phe$_1$...Thr$_{166}$**
    Met$_1$...Gly$_{395}$End
                ▲   ▲   ▲
                                                           prM   80%E
        Signalase Kex2p   DPAP

20
The location of the signalase, Kex2p, and DPAP cleavage
sites involved in the processing of the MFα leader
peptide are indicated (See Example 1 of this application
for a detailed explanation). The dengue sequences are
25 indicated in bold. The Phe$_1$ and Thr$_{166}$ residues are the
N-terminal and C-terminal amino acid residues of prM,
respectively. The Met$_1$ residue is the N-terminal amino
acid of the envelope glycoprotein and Gly$_{395}$ is residue
395 from the amino terminal end of the envelope
30 glycoprotein.
        The pLS6prM80%E plasmid was transformed into
Saccharomyces cerevisiae strain GL43 (MATa *ura3-52 trp1Δ1
pep4:URA3*) and screened for 80%E expression as described
in Example 1. Proteins secreted into the culture medium
35 as well as total cellular proteins were treated with
Endoglycosidase H$_f$ (EndoH$_f$, New England Biolabs, Beverly,
MA) prior to analysis by SDS-PAGE followed by Coomassie
Blue staining or Western blot immunoprobing. A novel
secreted protein could not be identified either on

Coomassie Blue stained SDS-PAGE gels or Western blots
probed with anti-DEN2 hyperimmune mouse ascitic fluid
(HMAF; from R. Putnak, Walter Reed Army Institute for
Research) or anti-DomB antiserum (see Example 6).
5   Western blot analysis of total cellular protein revealed
a HMAF immunoreactive band of approximately 90 kD
suggesting that the recombinant product had not been
processed to prM and E. Probing of companion Western
blots with polyclonal antisera that recognized the MFα
10   leader peptide (from J. Rothblatt, Dartmouth University)
confirmed that the product recognized by the anti-DEN2
HMAF was identical to that recognized by anti-MFα serum,
demonstrating that the MFα$_L$-prM80%E fusion protein was
not processed into its individual components (MFα$_L$, prM,
15   and 80%E).

The unsuccessful processing of E from prM in
the MFα$_L$-prM80%E fusion protein may be an obstacle to the
proper folding and secretion of E. To assess whether the
optimized dengue signal sequence (see Example 16)
20   facilitated the processing of the envelope protein at the
prM-E junction, the altered E signal sequence from
pLS6prM(mutSS)100E-TGA was introduced into pLS6prM80%E to
create plasmid pLS6prM(mutSS)prM80%E. This procedure
replaced the native E signal sequence (Pro-Ser-Met-Thr$_{-1}$-
25   Met$_{+1}$) with the optimized E signal sequence (Gly-Ala-Gln-
Ala$_{-1}$-Gln$_{+1}$).

Plasmid pLS6prM(mutSS)100E-TGA was obtained by
homologous replacement of a SmaI-SacI fragment between
plasmids pAlterSmaH3prME(mutSS) (see Example 14) and
30   pLS6prM100E. DNA sequencing of pLS6prM(mutSS)100E-TGA
identified an unintended TGA stop codon within E
downstream of the mutated secretion signal. To transfer
the altered secretion signal encoding sequence to
pLS6prM80%E and to separate the cDNA fragment containing
35   the altered secretion signal of E from the TGA stop
codon, a BglII-EcoNI fragment from pLS6prM(mutSS)100E-

TGA, encompassing prM and the first 430 nucleotides of E and lacking the TGA stop codon, was transferred to plasmid pLS6prM80%E which had been similarly digested to yield the expression plasmid pLS6prM(mutSS)80%E. The

5 sequence of the expression plasmid was confirmed by restriction digestion and DNA sequence analysis. The nucleotide and predicted amino acid sequences of the MFα_L-prM junction are identical to the sequences listed above.

10   As described for the nonmutagenized plasmid, the DNA was transformed into the *S. cerevisiae* GL43 strain and transformants were selected based upon their ability to grow on unsupplemented minimal medium (see Example 11). Transformants were cultured, induced, and

15 evaluated as described above for the nonmutated MFα_L-prM80%E transformants. Proteins secreted into the culture medium as well as total cellular proteins were treated with EndoH$_f$ prior to separation by SDS-PAGE. Protein gels were analyzed by Coomassie staining and

20 immunoprobing of Western blots. SDS-PAGE analysis of concentrated culture medium failed to identify a novel Coomassie staining band. Immunoprobing with anti-DEN2 HMAF and anti-DomB antiserum, however, revealed a small amount of processed immunoreactive E protein in the

25 medium. The size of the immunoreactive protein (approximately 50 kD) was similar to the secreted protein from pLS6-80%E expression vector. Evaluation of intracellular expression of the fusion protein containing the optimized secretion signal by SDS-PAGE and Western

30 blot demonstrated that the transformed cells produce immunoreactive product recognized by anti-DEN2 HMAF and anti-DomB antiserum. Unlike the immunoreactive product seen in pLS6prM80%E transformants, the immunoreactive band found in pLS6(mutSS)prM80%E transformants was not

35 recognized by MFα_L anti-serum suggesting that processing had occurred at the prM-E junction. Thus, the mutagenesis of the signalase cleavage site resulted in

greatly enhanced processing of the MFα_L-prM80%E product
at the prM-E junction.

Example 16
Construction of expression vector pPIC9-prM(mutss)80%E
and secretion of 80%E by *P. pastoris* expressing MFα_L-
prM(mutss)80%E

5

The expression vector constructed to express
preMembrane-mutated secretion signal-80%Envelope
10   (pPICprM(mutSS)80%E) in *P. pastoris* from a single
continuous open reading frame utilized the DEN-2 PR159/S1
prM and E gene sequences described above (Example 14).
The plasmid, pPIC9-prM(mutSS)80%E, was constructed by
transferring a prM(mutSS)80%E fragment from the *S.*
15   *cerevisiae* expression plasmid pLS6prM(mutSS)80%E into
pPIC9. The *P. pastoris* expression vector pPIC9 (Example
4) and the *S. cerevisiae* expression vector pLS6 (Example
2) both use the MFα prepropeptide leader (MFα_L) sequence
to direct the secretion of expressed proteins, and the
20   two MFα_L sequences share an *XhoI* restriction endonuclease
site, encoding amino acids leucine and glutamic acid,
just upstream of the Kex2 protease site. The transfer of
the dengue cDNA fragment made use of this *XhoI* site.

Prior to transferring the prM(mutSS)80%E cDNA
25   fragment, sequences encoding extraneous amino acids and
an extraneous *XhoI* site at the MFα_L-prM fusion were first
removed. This was accomplished by digesting pLS6-
prM(mutSS)80%E with restriction endonuclease *XhoI* and
XmaI into which was ligated a synthetic oligonucleotide
30   duplex (5'-TCGAGAAGAGAGAAG-3' and 5'-CCGGCTTCTCTCTTC-3').
This manipulation deleted the extraneous *XhoI* site, the
XmaI site, and six extraneous codons and preserved the
MFα_L proteolytic processing sites and the *XhoI* site
within MFα_L required for the cDNA fragment transfer from
35   pLS6 to pPIC9. The nucleotide and predicted amino acid

sequence at the MFα$_L$-prM fusion junction from pLS6-
prM(mutSS)80%E and pLS6Δ-prM(mutSS)80%E are:

MFα$_L$-prM junction of pLS6-prM(mutSS)80%E

5   _XhoI_   _XhoI_   _XmaI_

```
     XhoI                              XhoI   XmaI
ATG ...CTC GAG AAA AGG GAG GCC TTTAGATCTCGAGTACCCGGGACCATG TTT ..
Met ...Leu Glu Lys Arg Glu Ala PheArgSerArgValProGlyThrMet Phe₁..
              ▲               ▲
            Kex2p           DPAP
```

10

MFα$_L$-prM junction of pLS6Δ-prM(mutSS)80%E


```
     XhoI
ATG ...CTC GAG AAA AGG GAG GCC GGGACCATG TTT ..
Met ...Leu Glu Lys Arg Glu Ala GlyThrMet Phe₁..
              ▲        ▲
            Kex2p    DPAP
```

20

      To construct the clone pPIC9-prM(mutSS)80%E, a
XhoI-SalI fragment encoding prM(mutSS)80%E sequences was
obtained from pLS6Δ-prM(mutSS)80%E and was inserted into
the pPIC9 vector that had been digested with XhoI. The
25 genetic integrity of the expression plasmid, pPIC9-
prM(mutSS)80%E, was confirmed by restriction digestion.
      The pPIC9-prM(mutSS)80%E expression vector was
transformed into spheroplasts of P. pastoris strain GS115
(his4), and His⁺ transformants were selected for their
30 ability to grow on minimal medium without histidine
supplementation. The transformants were screened for
expression and secretion of 80%E as described in Example
12. No unique Coomassie staining bands were detected in
the culture medium of either nonEndoH$_f$ or EndoH$_f$ treated
35 samples (similar to that observed for culture medium from
pPIC9-80%E transformants - see Example 12). Western
immunoblots of proteins from the culture medium probed
with anti-domain B serum detected multiple bands with a
diffuse area of reactivity ranging from 50 to 90 kD in
40 the nonEndoH treated samples and a unique band of
approximately 50 kD in the EndoH treated samples. No
immunoreactive polypeptides were detected by the anti-

domain B serum in negative control samples. Various amounts of the corresponding immunoreactive protein were detected in EndoH treated samples of cellular proteins. We estimate that the amount of secreted 80%E in the

5   culture medium is less than 1% of the total amount of secreted protein based on the observation that no polypeptide of the appropriate molecular weight was detected by Coomassie staining of EndoH$_f$ treated samples.

10   Example 17

Construction of pMttbns-prM80%E and Secretion of 80%E by
Drosophila melanogaster Schneider cells Expressing tPA$_L$-
prM80%E

For expression of DEN-2 PR159/S1 preMembrane

15   protein amino acids 1-166 and Envelope glycoprotein amino acids 1-395 as a single continuous open reading frame in Drosophila melanogaster Schneider 2 tissue culture cells, DNA sequences encoding these proteins were obtained by digestion of the p48BSprM80%E clone (described in Example

20   14) with the restriction enzymes BglII and SalI. This fragment was cloned into the unique BglII and XhoI restriction sites of plasmid pMttΔXho (described in Example 13). The cloning junctions of the resulting gene fusion expression vector, pMttprM80E, were confirmed by

25   restriction endonuclease digestion and DNA sequence analysis. The partial nucleotide and predicted amino acid sequences of the tPA$_L$-prM80E fusion gene are:

```
ATG ............ GGAGCCAGATCTCGAGTACCCGGGACCATG TTT ..ACA   ATG ..GGA   TAA
```
30 Met-20 aa- -11 aa-GlyAlaArgSerArgValProGlyThrMet Phe$_1$..Thr$_{166}$ Met$_1$..Gly$_{395}$
END
        pre▲pro-tPA▲   prM   80%E

35   The tPA pre- and propeptide regions are delineated by pre▲ and pro-tPA▲, respectively, and the dengue sequences are indicated in bold type. The Phe$_1$ and Thr$_{166}$ residues are the N-terminal and C-terminal amino acid residues of prM, respectively. The Met$_1$ residue is the N-terminal

amino acid of envelope glycoprotein and Gly_{395} is residue 395 from the amino terminal end of the envelope glycoprotein.

As described previously in Example 13,

5   Schneider 2 cells were cotransfected with pMttprM80%E DNA at ratios of 1:1, 5:1, and 20:1 relative to pCOHygro DNA. Transformants were induced with 200μM $CuSO_4$ and expression of prM80%E was examined at various times after induction. Proteins secreted into the culture medium as well as

10   cellular proteins were separated by SDS-PAGE. Protein gels were analyzed by both Coomassie Blue staining and immunoprobing of corresponding Western blots. Analysis of Coomassie Blue-stained SDS-PAGE gels identified a novel band of approximately 50 kilodalton in all

15   transfectants. This novel band was also recognized by anti-DEN-2 HMAF in Western blot analysis. This ~50 kD immunoreactive band is roughly the same size as the secreted EndoH-treated product from pLS6-80%E transformed yeast cells (Example 11) and slightly smaller than the

20   secreted 80%E from pMttbns80%E-transformed *D. melanogaster* Schneider cells (Example 13), suggesting the Envelope protein had been processed away from the preMembrane protein. (The size discrepancy between 80%E secreted by pMtt80%E and pMttprM80%E Schneider cells is

25   discussed in Example 13.) Polyclonal antisera to the pr portion of prM (from Peter Wright, Monash University, Australia) did not recognize the ~50 kD protein, confirming that the 80%E produced in the transfected cells was processed from prM. In fact, no evidence of a

30   higher molecular weight band that might correspond to unprocessed prM80%E was detected in any sample, suggesting that the proteolytic processing of prM from E is extremely efficient in Schneider cells. The fate of the prM portion of the fusion remains unresolved as no

35   distinct immunoreactive band was detected by probing with the anti-pr antisera.

The secreted 80%E glycoprotein was partially
purified (judged by the presence of a single major band
on a sliver stained SDS-PAGE gel) and its N-terminal
amino acid sequence was determined. To purify the
5   secreted glycoprotein, culture medium was concentrated
and buffer exchanged against 20 mM succinate pH 5.7. The
buffer exchanged material was loaded onto a CM-BioGel
column and eluted in 150 mM NaCl. The 150 mM NaCl eluant
was separated on an SDS-PAGE gel and electro-transferred
10   to Immobilon-P membrane (Millipore). The 80%E band was
excised, and the N-terminal amino acids were determined
by Edman sequencing. Two amino acid sequences were
obtained. One, Met-Xxx-Xxx-Ile-Gly-Ile, had an individual
residue yield of 7.9 -10.0 picomoles, while the other,
15   Val-Xxx-Val-Gly-Ala-Val, had a 3.2- 4.2 picomole yield.
Incomplete reduction of the Cys at position three may
account for lack of its detection. the first sequence is
consistent with the expected sequence, Met-Arg-Cys-Ile-
Gly-Ile, supporting the interpretation that the ~50 kD
20   secreted immunoreactive glycoprotein is correctly
processed 80%E of DEN-2.
                 Sensitivity of the secreted 80%E to
endoglycosidases was evaluated by molecular weight shift
of the protein in SDS-PAGE and Western immunoblots
25   following endoglycosidase treatment. Resistance of the
secreted 80%E to Endoglycosidase H_f (Endo H_f; New England
Biolabs) and sensitivity to N-glycosidase F (PNGase F;
New England Biolabs) digestion indicated that the
secreted product contains N-linked glycosylation, and
30   that the glycosylation is probably complex and is neither
high mannose nor hybrid in composition.
                 The secreted protein is one of the predominant
proteins in the unconcentrated medium, comprising as much
as 20% of the total secreted protein. Estimates of the
35   concentration of the 80%E product in unconcentrated
medium based upon sandwich ELISA assays (described in
detail in Example 7) and Coomassie blue staining range

from 3-16 μg/ml depending on the preparation.
Immunoblots probed with polyclonal anti-dengue 2
hyperimmune mouse ascites fluid (DEN-2 HMAF; from R.
Putnak, WRAIR) demonstrated that the amount of secreted
5   80%E produced by the transfectants increased over time
from day 1 post induction to 7 days post induction. The
amount of 80%E detected intracellularly in the
transfectants correlated with the cotransfection ratio,
but the increase in intracellular 80%E with time was not
10   as dramatic as for secreted 80%E, suggesting efficient
secretion of 80%E and accumulation in the medium.

Example 18
Induction of anti-Dengue 2 antibodies in mice by _Pichia_
15   _pastoris_-secreted 80%E
         P. pastoris cells transformed with pPIC-80%E
(described in Example 12) were induced with 0.5% methanol
and the medium was collected after 40 hours of induction
(for additional details on culture conditions see Example
20   12). The medium was filtered through a 0.5 μm low
protein binding filter (Opticap, Millipore), then buffer
exchanged with phosphate buffered saline (10 mM sodium
phosphate, 2 mM potassium phosphate, 0.15 M sodium
chloride, and 27 mM potassium chloride, pH 7.5) and
25   concentrated approximately 40 fold using a combination of
tangential flow (Minitan; Millipore) and centrifugal
ultrafiltration (Centriprep 30; Amicon). The
concentrated medium was analyzed by Western blot and
assayed by sandwich ELISA (described in detail in Example
30   7) prior to injection into mice. Five week-old Swiss
Webster outbred mice (Simonsen) were immunized by
intraperitoneal (I.P.) injection with 100 μg total
protein of the crude concentrated 80%E medium with or
without complete Freund's adjuvant. Controls for this
35   experiment included a negative control medium prepared
from a nonrecombinant _P. pastoris_ culture as described
above for the 80%E medium. Protein precipitation was

observed during the concentration of the negative control
medium, consequently the final protein concentration of
the concentrated medium was lower than that from the 80%E
medium.   (For this reason, 12.5 μg of total protein in
5   Freund's complete adjuvant was used for immunization with
the negative control medium.)   Additional controls
included saline and KLH-domain B, a recombinant dengue
product previously shown to induce neutralizing
antibodies (Example 8), both were administered with
10   Freund's complete adjuvant. The mice received three I.P.
boosts consisting of one half the priming dose with or
without Freund's incomplete adjuvant, and consistent with
the specific priming immunization. The boosts were
administered at two week intervals.
15   Following the second boost, the animals were
test bled (tail vein) and the immune response was
monitored using an indirect ELISA. In the ELISA assay,
plates were coated with a bovine serum albumin (BSA)-
domain B conjugate, blocked with BSA, and serial
20   dilutions of the mouse sera were then incubated with the
coating antigen. Alkaline phosphatase-labeled goat
anti-mouse IgG was used as the secondary detecting
antibody, and the color development upon addition of an
alkaline phosphatase chromogenic substrate was monitored.
25   The ELISA titer is that which corresponded to the highest
dilution of serum resulting in an optical density two-
fold above background (reactivity of the serum against
BSA only). Following the third boost, the animals were
bled-out and the serum was tested for anti-DEN2
30   responsiveness by indirect ELISA and the plaque reduction
neutralization assay (PRNT). In the PRNT assay, the
mouse sera were serially diluted in Eagles minimal
essential medium (EMEM; BioWhittaker) supplemented with
10% fetal bovine serum (FBS; Hyclone) and mixed with 100
35   plaque forming units (pfu) of DEN2 virus (New Guinea C
strain, from Robert Putnak, WRAIR). After allowing one
hour for binding and neutralization of the virus, the

serum-virus mixtures were plated onto susceptible monkey
kidney monolayers (Vero cells, from Robert Putnak, WRAIR)
plated in EMEM (BioWhittaker) supplemented with 10% FBS
(Hyclone) in 6 well tissue culture dishes (Corning). The

5   cells were overlaid with 0.9% agarose (Seakem; FMC) in
EMEM supplemented with 5% FBS and viral cytopathic effect
was allowed to develop for 6-7 days. The resulting viral
plaques were stained with 0.012% neutral red (Sigma) in
1% agarose (Seakem; FMC). The number of plaques in each

10 cluster were counted and compared to a no-serum viral
control. The PRNT$_{80}$ was the highest dilution of serum
that resulted in an 80% reduction in the number of
plaques from a given viral inoculum. Results from the
ELISA and PRNT assays are summarized in Table 4. The *P.*

15 *pastoris* expressed 80%E induces a potent anti-DEN2
response in mice, with ELISA titers of up to 1:102,400.
The titers obtained in the presence of adjuvant exceeded
those obtained without adjuvant. The lack of a strong
virus neutralizing response (PRNT$_{80}$ titers, Table 4) may

20 simply reflect the crude nature of the immunogen, as
similar results were obtained when crude domain B was
used as an immunogen (see Example 8).

Table 4					
Induction of Anti-DEN2 Immune Response in Mice Immunized with *P. pastoris*-expressed 80%E					
mouse	antigen	adjuvant	30 titer ELISA	40 titer ELISA	40 titer PRNT$_{80}$
30-1	Saline	Freund's	<1:50	<1:100	
30-2			<1:50	<1:100	
30-3			<1:50	<1:100	
30-4			<1:50	<1:100	
30-5			<1:50	<1:100	
32-1	KLH- DomB "26"Tg	Freund's	>1:6400	1:25600	
32-2			>1:1600	1:102,400	
32-3			<1:100	1:100	
32-4			>1:6400	1:102,400	
32-5			<1:100	<1:100	
34-1	12.5 Tg Pichia negative control medium	Freund's	<1:100	<1:100	
34-2			<1:100	1:100	
34-3			<1:100	<1:100	
34-4			<1:100	<1:100	
34-5			<1:100	1:100	
36-1	100 Tg Pichia 80%E total medium	Freund's	>1:6400	1:25,600	<1:10
36-2			1:6400	1:25,600	<1:10
36-3			>1:6400	1:25,600	<1:10
36-4			1:100	1:100	<1:10
36-5			1:6400	1:102,400	<1:10
37-1	100 Tg Pichia 80%E total medium	None	<1:100	1:100	
37-2			1:1600	1:6400	
37-3			1:100	1:400	
37-4			1:400	1:6400	
37-5			1:100	<1:100	

Example 19
Induction of dengue virus-neutralizing antibodies by
immunizing mice with 80%E secreted by *Drosophila*
melanogaster Schneider cells expressing tPA$_L$-prM80%E and
tPA$_L$-80%E

Schneider cells, transformed with pMtt-prM80%E
and pMtt-80%E expressing the tissue plasminogen activator
leader fusion proteins tPA$_L$-prM80%E and tPA$_L$-80%E,
respectively (Described in detail in Examples 17 and 13,
respectively), were cultured in serum-free medium
(Excell; JRH Biosciences) and induced by addition of
CuSO$_4$ to a final concentration in the culture medium of
0.2 mM (see examples 13 and 17 for more detail on culture
conditions). The cells were maintained in inducing
medium for seven days prior to harvesting. The cells
were removed by centrifugation at 1000 X G in a Beckman
TJ-6 refrigerated centrifuge and the media were filtered
through a 0.2 μm cellulose acetate filter (Nalgene). The
media containing the recombinant 80%E were concentrated
20-fold using centrifugal concentrators (Centriprep 30;
Amicon) and assayed by ELISA (described in detail in
Example 7) and Western immunoblots prior to their use as
an immunogen in mice. Negative control medium, derived
from Schneider cells transformed with pCOHygro only (See
Example 13), was produced as described above. In two
series of immunizations, outbred Swiss Webster mice
(Simonsen) were immunized intraperitoneally (I.P.) with
100 μg total protein in Freund's complete adjuvant.
Control animals were immunized with 100 μg of total
protein from the concentrated negative control medium, 80
μg of purified *Saccharomyces*-expressed domain B (see
Example 8), or saline only, each in Freund's complete
adjuvant. In the first series of immunizations, the
mice received three I.P. boosts, consisting of one half
the priming dose in Freund's incomplete adjuvant, at two

week intervals. In the second series of I.P. immunizations, the mice received two boosts, each at one month intervals.

5   Following the first and second boosts, the animals were test bled (tail bleed) and the immune response was monitored using an indirect ELISA as described in example 18. Following the final boost, the animals were bled out and the sera were tested for anti-DEN2 responsiveness by indirect ELISA and PRNT as

10   described in Example 18. Results for the ELISA and PRNT assays are summarized in Tables 5 and 6. In both series of immunizations, the mice immunized with the crude media containing 80%E, expressed cotranslationally with prM or independently without prM, developed high titer, virus-

15   neutralizing antibodies. These titers are higher than any previously reported titers for any immunogen produced from any flavivirus, suggesting the utility of these immunogens as efficacious vaccine candidates.

| Table 5 |||||||
| Immune Response of Mice Immunized with Crude *Drosophila* Media Containing Dengue 2 Virus 80%E Expressed as a prM80%E Fusion |||||||
mouse	antigen	adjuvant	20 titer[a] ELISA	30 titer[b] ELISA	Final titer[c] ELISA	PRNT₈₀ titer[c]
20-1	Saline	Freund's	< 1:50	< 1:50	< 1:100	< 1:10
20-2			< 1:50	< 1:50	< 1:100	< 1:10
20-3			< 1:50	< 1:50	< 1:100	< 1:10
20-4			< 1:50	< 1:50	< 1:100	< 1:10
20-5			< 1:50	< 1:50	< 1:100	< 1:10
21-1	pCoHygro	Freund's	< 1:50	< 1:50	< 1:100	< 1:10
21-2			< 1:50	< 1:50	< 1:100	< 1:10
21-3			< 1:50	< 1:50	< 1:100	< 1:10
21-4			< 1:50	< 1:50	< 1:100	< 1:10
21-5			< 1:50	< 1:50	< 1:100	< 1:10
22-1	prM80%E	Freund's	1:3200	> 1:25,600	1:102,400	1:2560
22-2			> 1:800	> 1:6,400	1:25,600	1:2560
22-3			> 1:200	> 1:1,600	1:25,600	1:2560
22-4			< 1:50	> 1:102,400	> 1:102,400	1:2560
22-5			1:3200	> 1:25,600	> 1:25,600	1:640

[a]Determined following the 2nd injection.
[b]Determined following the 3rd injection.
[c]Determined following the 4th and final injection.

5

mouse	antigen	adjuvant	20 titer[a] ELISA	3/ titer[b] ELISA	Final titer[c] PRNT₈₀
25-1	Saline	Freund's	<1:50	>1:50	<1:10
25-2			<1:50	>1:50	<1:10
25-3			<1:50	>1:50	<1:10
25-4			<1:50	>1:50	<1:10
25-5			<1:50	>1:50	<1:10
27-1[d]	80Tg Purified Sacchro DomB	Freund's	1:1600	1:102,400	<1:100
27-2			1:100	1:6400	1:10
27-3			>1:6400	1:102,400	<1:100
27-4			>1:6400	1:409,600	1:40
27-5			>1:6400	1:409,600	<1:500
28-1	100Tg Drosophila prM80%E total medium	Freund's	DEAD	NT	NT
28-2			>1:6400	1:102,400	1:8000
28-3			>1:6400	1:409,600	1:8000
28-4			>1:6400	1:102,400	1:4000
28-5			>1:6400	1:102,400	1:1000
29-1	100Tg Drosophila 80%E total medium	Freund's	1:6400	1:6400	1:500
29-2			1:6400	1:102,400	1:4000
29-3			<1:100	1:25,600	1:1000
29-4			>1:6400	1:25,600	1:8000
29-5			>1:6400	1:102,400	1:4000

Table 6
Immune Response of Mice Immunized with Crude *Drosophila* Media Containing Dengue 2 Virus 80%E Expressed with or without prM

[a]Determined following the 2nd injection.
[b]Determined following the 3rd injection.
[c]Determined following the 4th and final injection.
[d]mouse 27-1 did not receive second boost.
NT - not tested.

Example 20

Protection from dengue virus challenge by immunizing mice
with 80%E secreted by _Drosophila melanogaster_ Schneider
cells expressing tPA_L_-prM80%E or tPA_L_-80%E.

5   Schneider cells, transformed with pMtt-prM80%E
and pMtt-80%E expressing the tissue plasminogen activator
leader fusion proteins tPA_L_-prM80%E and tPA_L_-80%E
respectively (described in detail in Examples 17 and 13,
respectively), were cultured in serum-free medium
10   (Excell; JRH Biosciences) and induced by addition of
$CuSO_4$ to the culture medium at final concentration of 0.2
mM (see examples 13 and 17 for additional details on
culture conditions). The cells were maintained in
inducing medium for seven days prior to collecting the
15   medium. The cells were removed by centrifugation at 1000
X G in a Beckman TJ-6 refrigerated centrifuge and the
media were filtered through a 0.2 µm cellulose acetate
filter (Nalgene). The media were concentrated 20-fold
using centrifugal ultrafiltration (Centriprep 30, Amicon)
20   and assayed by ELISA and Western immunoblots prior to use
as an immunogen in mice. Negative control medium,
derived from Schneider cells transformed with pCOHygro
only (see Example 13), was produced as described above.
These immunogens were used to immunize 10-13 day old
25   Balb/c mice (Jackson Labs). A total of 0.1 ml of each
preparation (corresponding to 70 µg total protein from
negative control medium, 230 µg total protein from tPA_L_-
80%E medium, and 150 µg total protein from tPA_L_-prM80%E
medium) were used to subcutaneously inoculate groups of
30   10 mice each, using Alum as adjuvant. An identical
second dose was administered 14 days later as a booster.
One week after the second dose the mice were challenged
with an intracranial injection of 100 LD_{50} units of DEN2
New Guinea C strain (10^5 pfu/injection).
35   The morbidity and mortality of the mice were
monitored for 17 days post challenge. Six days after

challenge the mice immunized with the negative control
medium began exhibiting symptoms of infection including
ruffled fur, hindlimb paralysis, wasting, and death.
Eight of the 10 negative control mice were dead by day 12
5   post-challenge and the remaining two mice recovered by
day 15 post-challenge. In contrast, of the 10 mice
immunized with the tPA$_L$-80%E, only four exhibited any
symptoms of infection, and eight of 10 survived the
challenge. Similarly, nine of 10 mice immunized with
10   tPA$_L$-prM80%E survived the challenge, although seven of
these mice exhibited mild symptoms of infection during
the monitoring period. These survival data are
illustrated in Figure 13, and show that both *Drosophila*-
expressed 80%E antigens efficiently protected mice from
15   viral challenge. These results emphasize the utility of
the *Drosophila* cell expressed 80%E dengue immunogens as
vaccine candidates.

                    Example 21
20   Construction of DEN-2 N-terminal 60%E and
                  prM60%E cDNA Fragments
          A subclone encoding the N-terminal 60% of E was
constructed using p29GEB24PS and p29D280E. Example 1 of
the parent application --- describes construction of
25   p29GEB24PS. Plasmid p29GEB24PS holds a *Bam*HI fragment
insert containing, in part, DEN-2 E sequences
(nucleotides 1696-2121) starting at a DEN-2 genomic *Bam*HI
site and ending with the Gly395 codon, followed
immediately by two stop codons and a *Sal*I site.
30   Oligonucleotide mutagenesis had deleted codons for Leu292
through Lys295 (nucleotides 1810-1821) and had inserted
two stop codons and a *Sal*I site immediately following the
Lys291 codon (see Figure 4 and Example 1).
          The N-terminal 80%E insert in p29D280E was then
35   converted to a 60%E insert by replacing a restriction
fragment encoding the 3' end of 80%E with a restriction
fragment from p29GEB24PS encoding the 3' end of 60%E. To

accomplish this, DNA of p29GEB24PS was digested with
*Bam*HI, the ~590 bp *Bam*HI fragment was isolated by agarose
gel electrophoresis and then digested with *Sal*I, and
finally the 119 bp *Bam*HI-*Sal*I fragment released from the
5   ~590 bp *Bam*HI fragment and containing dengue nucleotides
1696-1809 was isolated by agarose gel electrophoresis and
ligated into p29D280E prepared as follows. Plasmid
p29D280E was digested with *Bam*HI, which cuts the *Bam*HI
site (dengue nucleotides 1696-1701) within 80%E, and with
10   *Sal*I, which cuts immediately 3' of 80%E and also within
the vector, pBR322, 422 base pairs distal to the 3' end
of the 80%E fragment. Following ligation, the desired
product, a plasmid containing the cDNA encoding the
N-terminal 60% of E in pBR322 (p29D260E), was recovered
15   by transformation of *E. coli* with the ligation mixture
and screening transformant colonies for plasmids of the
appropriate size and restriction digestion pattern.
Proper ligation of the *Bam*HI-*Sal*I fragment in p29D260E
was confirmed by DNA sequence determination.
20   To construct a cDNA encoding prM and the amino
terminal 60% of E (prM60%E), we used a strategy identical
to that used to construct prM80%E (Example 14). The
prM100%E plasmid, p29prME13, was digested with *Bam*HI and
*Sal*I to release the 794 bp 3' end fragment of E, which
25   was then replaced with the 119 bp *Bam*HI-*Sal*I fragment
encoding a 40% carboxy-end truncation of E from p29D260E.
The resulting truncated cDNA clone, p48BSprM60E, encodes
a prM-60%E fusion ending with Lys291 of E and was
confirmed by restriction digestion and DNA sequence
30   analysis.

Example 22

Construction of expression vector pLS6-60%E and secretion
of 60%E by *Saccharomyces cerevisiae* expressing MFα-60%E

35   An expression vector (pLS6-60%E) was
constructed for secretion of the N-terminal 60% (codons
1-291, 60%E) of the DEN-2 PR-159 S1 envelope glycoprotein

from *S. cerevisiae*. The 60%E DNA sequences were obtained
from plasmid p29D260E, described in example 21, by
restriction endonuclease digestion with both *Bgl*II and
*Sal*I. The released fragment was isolated by agarose gel
5 electrophoresis and subcloned into the *Bgl*II and *Sal*I
sites of pLS6, a yeast expression vector described in
example 2. The MFα_L-60%E fusion was made such that
processing of the MFα propeptide and trimming of a Glu-
Ala dipeptide results in 60%E with eight additional N-
10 terminal amino acids encoded by sequences present in the
multiple cloning site of the pLS6 vector and the E gene
PCR primer adapter (see below).


```
     ATG .....GCT......GAG GCC TTT AGA TCT CGA GTA CCC GGG ACC ATG ...AAA  TAA TAG
15   Met-18aa Ala-65aa Glu Ala Phe Arg Ser Arg Val Pro Gly Thr Met₁...Lys₂₉₁End
     End
          ▲         ▲        ▲                                          60%E
        Signalase  Kex2p   DPAP
```

20
Transcription of the gene fusion is driven by the *S.*
cerevisiae copper-inducible copper metallothionein (*CUP1*)
promoter.

After confirming the DNA sequence of the
25 ligated junctions of expression vector pLS6-60%E, the
recombinant DNA was transformed into *S. cerevisiae* strain
GL43 (*MATa trp1Δ1 ura3-52 pep4::URA3*; SmithKline Beecham)
according to standard protocols (Gutherie & Fink, eds.
1991; Rose et al, 1990). Transformants were selected by
30 their ability to grow on minimal medium (SD medium:
Guthrie and Fink, 1991) without tryptophan
supplementation.

In order to test for expression and secretion
of 60%E, several transformants were grown in small-scale
35 cultures (5 ml medium in 17x150 mm tubes). Single
colonies were used to inoculate SD medium and the
inoculated cultures were grown to saturation (overnight,
30°C, 220 rpm). 1×10^8 cells from the overnight culture
were used to inoculate 5 ml of minimal SD medium

supplemented with Casamino acids (2 g/l; Difco) and $CuSO_4$
(200 μM). This expression culture was fed with glucose
(4 g/l, final concentration) and sodium phosphate (pH
6.7, 20 mM, final concentration) at 24 hours post

5   inoculation and, after 48 hours of growth (30°C, 220 rpm,
in 17x150 mm tubes), was harvested by centrifugation.
Cell-free spent medium was buffer-exchanged with TEEN (50
mM Tris, 10 mM EDTA, 10 mM EGTA, 150 mM NaCl, pH 8.0) and
concentrated 200-fold using Centricon-30 (Amicon)

10 ultrafiltration. An extract of cellular protein was
prepared by lysing the yeast cells with vigorous
agitation in the presence of glass beads (425-600 μm) and
TEEN+PIC (TEEN with 1 μg/ml each of pepstatin and
leupeptin and 1 mM phenylmethylsulfonylfluoride) using a

15 Mini Beadbeater apparatus (BioSpec Products,
Bartlesville, OK). Samples were endoglycosidase H_f
digested according to the manufacturer's (New England
Biolabs, Beverly, MA) protocol prior to SDS-PAGE
analysis. Protein gels were Coomassie-stained directly

20 as well as Western blotted and immunoprobed using anti-
DEN2 HMAF.

Negative control yeast carrying the expression
vector without a Dengue gene insert secreted no proteins
recognized by anti-DEN2 HMAF, while pLS6-60%E medium

25 contained several immunoreactive species. The major band
presumably represents full-length 60%E since its apparent
molecular weight is approximately 10 kD less than that of
recombinant 80%E. A protein band comigrating with this
immunoreactive material was visible in Coomassie-stained

30 gels of proteins secreted by pLS6-60%E transformants, but
this band was absent in medium of negative control
transformants. The immunoblot of proteins secreted by
pLS6-60%E transformants evidenced a minor band of
apparent molecular weight 6-8 kD larger than 60%E; this

35 likely represents unprocessed MFα propeptide-60%E. The
cellular protein extract of pLS6-60%E transformants

contained many immunoreactive polypeptides not observed
in negative control cells; two of these match the
secreted products discussed above.

                        Example 23
   Construction of expression vectors pLS6-prM60%E and
   pLS6-prM(mutSS)60%E, expression of MFα_L-prM60%E and
   MFα_L-prM(mutSS)60%E in *Saccharomyces cerevisiae*, and
   secretion of 60%E by *Saccharomyces cerevisiae* expressing
                   MFα_L-prM(mutSS)60%E

       For expression of DEN-2 PR159/S1 premembrane
protein amino acids 1-166 and envelope glycoprotein amino
acids 1-291 as a single contiguous open reading frame in
S. cerevisiae, DNA sequences encoding these proteins were
obtained by digestion of plasmid p48BSprM60E (see Example
21) with the restriction enzymes *Bgl*II and *Sal*I. This
fragment was cloned into the pLS6 *S. cerevisiae*
expression vector (see Example 2) that had been digested
with *Bgl*II and *Sal*I. The structure of the resulting gene
fusion expression vector, pLS6-prM60%E, was confirmed by
restriction digestion and DNA sequence analysis. The N-
terminal MFα_L-prM fusion amino acid sequence of the MFα_L-
prM60%E fusion protein are identical to those described
for MFα_L-prM(mutSS)80%E fusions (Example 15), while the
C-terminal amino acid of the fusion protein is Lys_291 of
the dengue envelope glycoprotein.

       The pLS6-prM60%E plasmid was transformed into
S. cerevisiae strain GL43 (*MAT*a *ura3-52 trp1Δ1
pep4::URA3*) and screened for 60%E expression as described
in Example 11. Proteins secreted into the culture medium
as well as cellular proteins were treated with
endoglycosidase H_f (EndoH, New England Biolabs, Beverly,
MA) and separated by SDS-PAGE. Proteins were analyzed by
both Coomassie staining of polyacrylamide gels and
immunoprobing of Western blots. Similar to the
expression of secreted 80%E from pLS6-prM80%E (Example

15), secreted 60%E was not detected on Coomassie stained polyacrylamide gels nor Western blots. An immunoblot of intracellular protein confirmed that the construct (MFα$_L$-prM60%E) was expressed, but the fusion product had not been processed to prM and E (evaluation was performed as described in Example 15).

Because the dengue E signal sequence itself may limit processing of the prME fusion proteins, expression of MFα$_L$-prM(mutSS)60%E was evaluated. The *BglII-EcoNI* fragment from pLS6-prM(mutSS)100%E-TGA encoding the altered secretion signal peptidase cleavage site (see Example 15) was used to replace the homologous fragment from the pLS6-prM60%E to produce pLS6-prM(mutSS)60%E. The sequence of the expression plasmid was confirmed by restriction digestion and DNA sequence analysis. Plasmid pLS6prM(mutSS)60%E was transformed into the *S. cerevisiae* GL43 strain and transformants were selected as described in Example 11.

Transformants were cultured, induced, and evaluated as described in Examples 11 and 15. In contrast to the expression of MFα$_L$-prM60%E, Western blot analysis of total intracellular proteins from pLS6-prM(mutSS)60%E transformants demonstrated that the transformed cells produce an approximately 40 kilodalton product recognized by anti-DEN2 HMAF. For analysis of secreted proteins, media from induced cultures were concentrated, treated with endoglycosidase H$_f$, and analyzed on Western blots for E antigen. A small amount of processed 60%E could be identified in the culture medium upon immunoprobing with anti-DEN2 HMAF. Thus, the mutagenesis of the signalase cleavage site resulted in greatly enhanced processing of the MFα$_L$-prM(mutSS)60%E product at the prM-E junction which produced secretion of a processed 60%E from the MFα$_L$-prM(mutSS)60%E in *S. cerevisiae*.

Example 24
Construction of expression vector pPIC9-60%E and
secretion of 60%E by *P. pastoris* expressing MFα_L-60%E

     The expression vector constructed to secrete
5 60%E from *P. pastoris*, pPIC9-60%E, included the DEN-2 PR-
159 S1 envelope glycoprotein amino acids 1-291. As a
precursor to this 60%E expression vector, a modified
pLS6-60%E plasmid (pLS6-alt60%E), encoding a fusion
between MFα_L and the amino terminal 60% of the dengue
10 envelope, was constructed that encodes fewer nondengue
amino acids between the MFα_L and E segments. The 60%E
cDNA fragment from pLS6-alt60%E was then transferred to
pPIC9.

     The sequences in pLS6-alt60%E encoding the
15 dengue E protein amino terminus were derived from pLS6-
2x80E, which encodes a tandemly arrayed dimer of 80%E
that are linked by a synthetic linker peptide. To
convert pLS6-2x80E to pLS6-alt60%E, pLS6-2x80E was
digested with *EcoNI*, which cuts within the first member
20 of the 80%E dimer, and *SalI*, which cuts immediately
downstream of the dimer, thereby removing the 3' portion
of the first member of the 80E dimer and the second
member of the 80E dimer entirely. In its place was
ligated an *EcoNI-SalI* fragment from pLS6-prM(mutSS)60E
25 (see Example 23), encoding the 3' portion of 60%E, to
complete construction of pLS6-alt60%E.

     The minimizing of nondengue codons between MFα_L
and E, found in pLS6-2x80E, were preserved in pLS6-
alt60%E, and, subsequently, in pPIC9-60%E. The codons
30 between MFα_L and E were minimized, owing to use of the
XmaI site in p29D280E (*XmaI* is the first adapter
restriction enzyme site upstream of E; see Example 1) and
the StuI site in pLS6. To ligate the *XmaI* 5' end of E to
the *StuI* site in the MFα_L, the *XmaI* site was treated with
35 Klenow polymerase to make the end blunt during the
construction of pLS6-2x80E.

To transfer 60%E from pLS6-alt60%E to pPIC9,
pLS6-alt60E was digested by *Xho*I plus *Sal*I, which
released a fragment that includes a portion of the MFα
leader and the entire 60%E coding region. This fragment
5   was ligated with the *Pichia* expression vector pPIC9
(Invitrogen, San Diego, CA; described in Example 12) that
was previously digested with the restriction enzyme *Xho*I.
This ligation restored the complete MFα leader sequence
including the Kex2 cleavage site. The genetic integrity
10   of the resulting gene fusion expression vector, pPIC9-
60%E, was confirmed by restriction digestion and DNA
sequence analysis. The partial nucleotide and predicted
amino acid sequences of the MFα$_L$-60%E fusion gene are
shown below:

15

```
      ATG ........ GAG GCC GGG ACC ATG ...AAA   TAA
      Met-18aa-65aa Glu Ala Gly Thr Met₁...Lys₂₉₁ END
               ▲      ▲           ▲
          Signalase  Kex2p    DPAP
```

20

The location of the signalase and *Kex2* cleavage sites
which remove the pre and pro portions of the MFα leader
peptide, respectively, are indicated. The dengue
25   sequences are indicated in bold type. The Met_1 residue
is the N-terminal amino acid of the E glycoprotein and
Lys_{291} is residue 291 from the amino terminal end of the
envelope glycoprotein. The expression of a recombinant
product in *Pichia* from the pPIC9 vector is driven by the
30   methanol inducible promoter derived from the *Pichia*
pastoris aox1 (alcohol oxidase 1) gene.

The pPIC9-60%E expression vector was
transformed into spheroplasts of *P. pastoris* strain
GS115 (*his4*) and transformants were selected for their
35   ability to grow on minimal medium without histidine
supplementation. Strain GS115 and the protocol used for
transformation were obtained from Invitrogen (San Diego,
CA). Transformants were tested for their ability to

express and secrete 60%E by growing selected clones in
small cultures (5 ml). The transformants were grown to
saturation (24 to 36 hrs.) in BMGY medium (1% yeast
extract, 2% peptone, 100 mM potassium phosphate, pH 6.0,
5   1.34% yeast nitrogen base without amino acids, 4×10^{-5}%
biotin, 1% glycerol). The cells were collected by
centrifugation and suspended in one half the original
culture volume with BMMY (identical to BMGY except the
glycerol component of BMGY is replaced in BMMY with 0.5%
10   methanol) medium and cultured for 48 hrs. Proteins
secreted into the culture medium, as well as cellular
proteins, were treated with endoglycosidase H$_f$ (EndoH,
New England Biolabs, Beverly, MA) and separated by SDS-
PAGE. Western immunoblots probed with DEN-2 HMAF
15   indicated that the recombinants expressed significant
levels of 60%E. Protein gels analyzed by Coomassie
staining also showed strong levels of 60%E expression and
secretion.

20   Example 25
      Construction of expression vector pPIC9-prM(mutSS)60%E
      and secretion of 60%E by _P. pastoris_ expressing MFα$_L$-
                          prM(mutSS)60%E
          To construct clone pPIC9-prM(mutSS)60%E, a
25   strategy identical to that describe in Example 16 was
used. Clone pLS6-prM(mutSS)60%E (described in Example
23) was digested with restriction endonuclease _XhoI_ and
XmaI and sequences within the MFα$_L$ and dengue cloning
adapter were replaced by oligonucleotides to remove an
30   extraneous _XhoI_ site, to preserve a critical _XhoI_ site,
and to regenerate the Kex2 protease process site. The
XhoI-SalI prM(mutSS)60%E fragment from pLS6Δ-
prM(mutSS)60%E was ligated into the unique _XhoI_ site of
pPIC9. The nucleotide and amino acid sequences at the N-
35   terminus of the fusion protein are identical to that
shown in Example 16 for pLS6Δ-prM(mutSS)80%E. The

structure of the *Pichia* expression vector pPIC9-prM(mutSS)60%E was confirmed by restriction digestion and DNA sequence analysis.

The pPIC9-prM(mutSS)60%E expression vector was transformed into spheroplasts of *P. pastoris* strain GS115 (*his4*), and transformants were selected and evaluated as described in Example 12. After EndoH$_f$ treatment of secreted proteins, a novel Coomassie Blue-staining band could be detected by SDS-PAGE. Immunoprobing with anti-domain B serum of Western blots of proteins prepared from the culture medium detected a diffuse area of reactivity ranging from 40 to 90 kD in the nonEndoH treated samples and a unique band of approximately 40 kD in the EndoH$_f$ treated samples that corresponds to the band observed following Coomassie Blue staining.

Example 26
Construction of pMttbns-prM100%E, Expression of tPa-prM100%E by *Drosophila melanogaster* Schneider cells, and Induction of a Virus Neutralizing Response by Immunizing Mice with tPa-prM100%E Membrane Preparations

For expression of DEN-2 PR159/S1 preMembrane protein amino acids 1-166 and Envelope glycoprotein amino acids 1-495 as a single continuous open reading frame in *Drosophila melanogaster* Schneider 2 tissue culture cells, DNA sequences encoding these proteins were obtained by digestion of the p48BSprME13 clone (described in Example 14) with the restriction enzymes Bg1II and SalI. This fragment was cloned into the unique Bg1II and XhoI restriction sites of plasmid pMttΔXho (described in Example 13).

The cloning junctions of the resulting gene fusion expression vector, pMttprM100E, were confirmed by restriction endonuclease digestion and DNA sequence analysis. The partial nucleotide and predicted amino acid sequences of the tPA$_1$-prM100E fusion gene are:

```
ATG ............. GGAGCCAGATCTCGAGTACCCGGGACCATG TTT ..ACA     ATG ..GGA    TAA
Met-20 aa- -11 aa-GlyAlaArgSerArgValProGlyThrMet Phe₁..Thr₁₆₆ Met₁
..Ala₄₉₅END
        pre▲pro-tPA▲                                      prM        100%E
```

₅

The TPA pre- and propeptide regions are delineated by pre⸱
and pro-tPA⸱, respectively, and the dengue sequences are
indicated in bold type. The Phe₁ and Thr₁₆₆ residues are
₁₀ the N-terminal and C-terminal amino acid residues of prM,
respectively. The Met₁ residue is the N-terminal amino
acid of envelope glycoprotein and Ala₄₉₅ is residue 495
from the amino terminal end of the envelope glycoprotein
(the carboxy-terminal residue).

₁₅   As described previously in Example 13,
Schneider 2 cells were cotransfected with pMttprM100%E
DNA at ratios of 1:1, and 5:1, relative to pCOHygro DNA
and selected for growth in medium containing 300 μg/ml
hygromycin. Transfectants were induced with 200μM CuSO₄
₂₀ and expression of prM100%E was examined at various times
after induction. Proteins secreted into the culture
medium as well as cellular proteins were separated by
SDS-PAGE. Protein gels were analyzed by both Coomassie
Blue staining and immunoprobing of corresponding Western
₂₅ blots. Analysis of Coomassie Blue-stained SDS-PAGE gels
failed to reveal a unique protein band either
intracellularly or in the culture medium. However, a
novel band was recognized intracellularly by polyclonal
anti-dengue 2 hyperimmune mouse ascites fluid (anti-DEN-2
₃₀ HMAF, gift of R. Putnak, WRAIR) in Western blot analysis.
This ~60 kD immunoreactive band comigrated with viral
Envelope derived from dengue-2 infected mosquito C6/36
cells suggesting the recombinant 100%E protein had been
processed away from the preMembrane protein. Polyclonal
₃₅ antisera to the pr portion of prM (from Peter Wright,
Monash University, Australia) recognized a ~20 kD
protein, which comigrated with viral prM, confirming that
the 100%E produced in the transfected cells was processed

from prM. In fact, no evidence of a higher molecular
weight band that might correspond to unprocessed prM100%E
was detected in any sample, suggesting that the
proteolytic processing of prM from E is extremely
5   efficient in Schneider cells. No 100%E was detected in
the culture medium indicating that 100%E remains anchored
in cell-associated membranes.

        Immunoblots probed with anti-DEN-2 HMAF
demonstrated that the amount of intracellular 100%E
10   produced by the transfectants increased over time from
day 1 post induction to 7 days post induction. The
amount of 100%E detected intracellularly in the
transfectants correlated with the cotransfection ratio.
Sensitivity of the intracellular 100%E to
15   endoglycosidases was evaluated by molecular weight shift
of the protein in SDS-PAGE and Western immunoblots
following endoglycosidase treatment. Partial resistance
of the recombinant 100%E to Endoglycosidase H_f (Endo H_f;
New England Biolabs) digestion indicated that the product
20   contains N-linked glycosylation, and that the composition
of the recombinant products probably represents a mixture
of complex, high mannose, and/or hybrid glycosylation.

        Schneider cells, transformed with pMtt-prM100%E
expressing the tissue plasminogen activator leader fusion
25   protein tPA_L-prM100%E were cultured in serum-free medium
(Excell; JRH Biosciences) and induced by addition of
$CuSO_4$ to a final concentration in the culture medium of
0.2 nM (see Examples 13 and 17 for more detail on culture
conditions). The cells were maintained in inducing
30   medium for seven days prior to harvesting. The cells
were harvested by centrifugation at 1000 X G in a Beckman
TJ-6 refrigerated centrifuge. The cells were washed one
time with phosphate buffer saline (PBS), resuspended in
PBS and disrupted with a tissue homogenizer. Undisrupted
35   cells were removed by centrifugation at 1000 X G in an
Eppendorf microcentrifuge. Cellular membranes were
pelleted at 10,000 X G in an Eppendorf microcentrifuge

and 100,000 X G in a Beckman L8-80M Ultracentrifuge. The
10,000 X G and 100,000 X G pellets were resuspended in
PBS and pooled for mouse immunization. Negative control
cells transformed with pCOHygro only (see Example 13)
5   were cultured, induced, and harvested as described for
the prM100%E-expressing cells. The immunogens were
assayed by Western blot prior to immunization of mice.
         Outbred Swiss Webster mice (Simonsen) were
immunized intraperitoneally (I.P.) with 75 µg total
10   protein of the prM100%E membrane preparation in Freund's
complete adjuvant. Control animals were immunized with
either 75 µg of total protein from the negative control
membrane preparation or with saline only, each in
Freund's complete adjuvant. The mice received three I.P.
15   boosts, consisting of one half the priming dose in
Freund's incomplete adjuvant, at two week intervals.
         Following the first and second boosts, the
animals were test bled (tail bleed) and the immune
response was monitored using an indirect ELISA as
20   described in Example 18. One week after the final boost,
the animals were bled out and the sera were tested for
anti-DEN2 responsiveness by indirect ELISA and PRNT as
described in Example 18. Results for the ELISA and PRNT
assays are summarized in Table 7. The mice immunized
25   with the membrane preparation containing 100%E developed
virus-neutralizing antibodies despite the crude nature of
the immunogen, suggesting the utility of these immunogens
as efficacious vaccine candidates.

Table 7						
Immune Response of Mice Immunized with Recombinant Dengue prM100%E Membrane Preparation						
mouse	antigen	adjuvant	20 titer[a] ELISA	30 titer[b] ELISA	final titer ELISA	PRNT80 titer[b]
20-1	Saline	Freund's	<1:50	<1:50	<1:100	<1:10
20-2			<1:50	<1:50	<1:100	<1:10
20-3			<1:50	<1:50	<1:100	<1:10
20-4			<1:50	<1:50	<1:100	<1:10
20-5			<1:50	<1:50	<1:100	<1:10
23-1	pCoHygro membrane	Freund's	<1:50	<1:50	<1:100	<1:10
23-2			<1:50	<1:50	<1:100	<1:10
23-3			<1:50	<1:50	<1:100	<1:10
23-4			<1:50	<1:50	<1:100	<1:10
23-5			<1:50	<1:50	<1:100	<1:10
24-1	prM100%E membrane	Freund's	1:200	1:1,600	>1:25600	1:320
24-2			<1:50	>1:1600	>1:1600	1:320
24-3			<1:50	>1:6000	>1:1600	1:80
24-4			<1:50	>1:6000	>1:25600	1:640
24-5			<1:50	<1:50	1:400	1:20

[a]Indirect ELISA titer using recombinant domain B as coating antigen.
[b]80% reduction of plaque titer of DEN-2 NGC virus on Vero cells.

Example 27

Immunoaffinity Purification of E and Subunits

Preparation of immunoaffinity columns:

Materials:
Cyanogen bromide (CNBr) activated sepharose 4B, lot 17-0430-01 (Pharmacia Biotech, Upsala, Sweden). (HiTrap) Bead size 45-165 μm .
1 mM HCl
Binding buffer (100 mM NaHCO3, 500 mM NaCl pH 8.3)
Low pH washing buffer (100 mM glycine, pH 2.5)
Equilibration buffer (100 mM phosphate, pH 7.2)
Monoclonal antibody 9D12, 4G2 or other (dengue envelope specific/reactive)

Procedure: 0.6g (~2 ml) of freeze-dried HiTrap was suspended in 5 ml of 1 mM HCl for 15 min. The gel was poured onto a sintered glass filter and washed with 120 ml of 1 mM HCl, followed by 10 ml of binding buffer.

Monoclonal antibody 9D12 was buffer exchanged into
binding buffer using a Centricon 30 (Amicon). The
concentration was estimated from its absorbance at 280 nm
(A_{280} of 1 mg/ml = 1.4). Approximately 20 mg of antibody
5 was added to washed matrix to a final volume of about 4
ml. The coupling reaction was allowed to proceed at 4°C
with end-over-end rotation. The antibody modified gel
was then poured into a polypropylene column (Biorad) and
washed. Washing involved sequential elution with
10 equilibration buffer - washing buffer - equilibration
buffer - binding buffer, for three cycles. The amount of
unbound antibody was estimated by measuring absorbance of
the eluants at 280 nm. Typically, about 18 mg of
antibody bound per 2 ml of gel. The column was stored at
15 4 °C until used. Prior to running media containing
protein, the column was washed with washing buffer
followed by equilibration buffer.

        Purification of proteins by immunoaffinity
chromatography. Chromatography was performed using low
20 pressure. Media or buffer was supplied to the antibody-
sepharose column by a peristaltic pump (Pharmacia or
Gilson). The effluent from the column was monitored for
absorbance at 280 nm using a Gilson 112 UV/vis detector
equipped with a flow through cell. Media containing
25 classical domain B, domain B+T, MFα80E and 80%E from
Drosophila cells was circulated through the
immunoaffinity column for antigen binding and washed with
phosphate buffer pH 7.0. The bound protein is eluted
with buffer at pH 2.5.

        Comparison of 9D12, 4G2 and 3H5 MAbs as
30 *affinity supports.* N-hydroxysuccinimide (NHS) activated
HiTrap columns (1 ml) were reacted with 5 mg of dengue
envelope-specific MAb 9D12, 4G2 or 3H5 according to
methods provided by the manufacturer. The columns were
35 then tested for their ability to purify DEN-2 80%E from
Drosophila cell culture medium, using the purification
procedure described above.. The 4G2 column had a greater

capacity than the 9D12 column, while the 3H5 column did
not bind significant amounts of DEN-2 80%E. Up to 150 μg
of 80%E could be bound by the 4G2 column, while the
capacity of the 9D12 column was about 70 μg.

5   *General protocol for purification of DEN-2 80%E
by 4G2 and 9D12 HiTrap columns.* The columns (1 ml or 5
ml bed volume) are perfused at a rate of 1 ml per minute
with media containing 80%E adjusted to pH 7.2. Following
washing with 10 mM phosphate buffer, pH 7.2, the antigen
10   is eluted with 100 mM glycine pH 2.5. The eluted product
is neutralized with 1 M Tris, pH 7.5 (final concentration
0.2 M), and NaCl is added to a final concentration of 150
mM. The sample is then concentrated by membrane
ultrafiltration in a Centricon 30 (Amicon).

15   *Concentration and purity determination.* The
concentration of purified 80%E was determined by ELISA,
using purified domain B as a standard. The purity of the
antigen was estimated by SDS-PAGE followed by Coomassie
staining or Western transfer, showing the immunoaffinity
20   purified 80%E antigens to be greater than 98% pure.

              Purification of (MFa$_L$)80%E from Pichia media.
(MFa$_L$)80%E was purified by immunoaffinity chromatography
using a MAb 9D12 column as described. For each run, 50
ml of yeast culture medium [~1 μg/ml (MFa$_L$)80%E] was
25   cycled for 3 hr through a 2 ml Sepharose column
containing 20 mg of MAb 9D12. The column was washed with
5 mM phosphate buffer, pH 7.0, until a baseline
absorbance at 280 nm was re-established. The bound
protein was eluted with 5 mM phosphate buffer, pH 2.5,
30   then concentrated 20-fold by evaporation using a SpeedVac
(Savant), and further concentrated by membrane
ultrafiltration using a Centricon 30 (Amicon). The
concentration of purified protein was estimated using a
MAb 9D12/rabbit anti-domain B sandwich ELISA. This assay
35   incorporates a purified *Pichia*-secreted domain B
standard, quantified by amino acid analysis (Protein and
Nucleic Acid Facility, Stanford CA). The molar amount of

(MFa$_L$)80%E eluted from the columns was determined by
comparison to a domain B standard curve. The
concentration of (MFa$_L$)80%E, was estimated by multiplying
the concentration of domain B equivalents by five, to
5   compensate for the mass/size difference between the
domain B standard and (MFa$_L$)80%E. Purity of the
(MFa$_L$)80%E product was assayed by SDS-PAGE analysis on a
12% gel. Prior to SDS-PAGE analysis, the protein was
treated with 5,000 units of Endo Hf (New England Biolabs)
10   for two hours to remove heterogeneous carbohydrates added
post-translationally to the molecule by the yeast. The
treated protein migrates as a single band of molecular
weight approximately 50,000 daltons.

        Approximately 0.6 mg of (MFa$_L$)80%E was
15   recovered by this method (~50% yield). The purified
subunit protein was stored in PBS at 4 °C, until used to
immunize mice.

                        Example 28
20   Ion-Exchange and Size Exclusion Chromatography
                Purification of Envelope Subunit
        Sterile-filtered, frozen stocks of supernatant
from *Drosophila* cell cultures grown in a bioreactor were
used as starting material for the purification procedure.
25   The sample was thawed then concentrated and diafiltered
using a tangential flow ultrafiltration device
(Millipore, Bedford, MA) containing membranes with
molecular weight cut-off of 10 kilodaltons. The sample
was first concentrated to approximately 200 ml then
30   diafiltered against 10mM Tris-succinate buffer (TSB), pH
8.1. The volume of TSB, pH 8.1, that was used for the
diafiltration was at least twice the starting sample
volume. Under the described conditions, 80%E was
retained by the membranes along with the majority of the
35   *Drosophila* proteins.

        The retentate from the diafiltration was loaded
directly onto a Fractogel DEAE (E-M Sciences, Gibbstown,

NJ) column (1.4cm id. x 5.5cm ht.) that was equilibrated
against TSB, pH 8.1. The sample was loaded at a flow-
rate of 1 ml per minute. Under these loading conditions,
all of the 80%E in the sample was bound to the anion-
5   exchange matrix. After the sample was loaded, the column
was washed with TSB, pH 8.1, until the absorbance at
280nm was zero. The column was then treated with TSB, pH
8.1, containing 100mM NaCl; this buffer eluted all bound
proteins including 80%E. The product from this step
10 contained large amounts of *Drosophila* contaminants;
however, a significant contaminating *Drosophila* protein
component as well as nonprotein components of the culture
media were removed.

The peak fractions from the 100mM NaCl elution
15 profile were then adjusted to pH 5.7 and loaded onto a
Fractogel-COO⁻ (E-M Sciences) column (1.4cm id. x 1.6cm
ht.) that was equilibrated against TSB, pH 5.7,
containing 100mM NaCl. The sample was loaded at a flow-
rate of 1 ml per minute. Most of the 80%E was bound to
20 the cation-exchange column, while many of the *Drosophila*
proteins were not. Once the sample was loaded, the
column was washed with TSB, pH 5.7 (no NaCl), followed by
TSB, pH 8.1(no NaCl). Bound 80%E was eluted with TSB, pH
8.1, containing 50 mM NaCl. The 80%E fraction from this
25 step was approximately 80% pure.

The Fractogel-COO⁻ column product was further
purified by gel filtration chromatography on a BioSep
3000 HPLC column (600x7.8mm, Phenomenex, Torrance, CA).
Phosphate buffered saline (25 mM phosphate, 100mM NaCl),
30 pH 6.8, was used as the running buffer at a flow rate of
0.5 ml per minute. Some of the 80%E was eluted in an
early peak fraction along with various *Drosophila*
contaminants; however, most of the material was eluted in
a later peak. The 80%E in the later fraction was >90%
35 pure.

Based on sandwich ELISA and SDS-PAGE analyses,
the recombinant Dengue protein retains complete

reactivity with MAb 9D12 that recognizes a
conformationally sensitive envelope epitope. Reactivity
with MAb 9D12 was retained during each step of the
purification process.

5

Example 29
Expression and secretion of DEN-2 NS1
from Drosophila cells

A fully sequenced DEN-2 PR159 S1 NS1 clone was
10 used as the starting construct. The plasmid clone,
p29D2NS1.2, contains a 1,055 bp fragment that corresponds
to nucleotides 2422 and 3477 of the parent DEN-2 genomic
map. The 5' end is flanked by a *Bgl*II site and the 3'
end is flanked by two stop codons and a *Sal*I site. This
15 *Bgl*II-*Sal*I fragment was cloned into the *Drosophila*
expression plasmid pMTT digested with the same two
enzymes. Two of the resulting recombinants were selected
for further characterization.

Two clones encoding the DEN2 NS1 protein,
20 pMttNS1.1 and pMttNS1.2, were transfected into S2 cells
and transfectants selected for hygromycin resistance.
The expression of the NS1 protein by the mixed
populations was evaluated by Western blot analysis.
Immunoreactive product was detected both intracellularly
25 and in the medium, with the vast majority of the product
being secreted into the medium. A corresponding
Coomassie blue staining band was seen on SDS-PAGE gels.
The secreted 80%E product appeared to accumulate in the
medium out to at least day 7 post induction. By Western
30 blot analysis, the NS1.2 transfectant population appears
to express at slightly higher levels than the NS1.1
transfectant population. The immunoreactive product is
recognized by anti-DEN2 HMAF and anti-NS1 MAb 7E11.
Digestion of the secreted product with Endoglycosidase H
35 (EndoH) and Peptide:N-glycosidase F (PNGase F)
demonstrated that the secreted product is sensitive to
both EndoH and PNGase F digestion and therefore is

glycosylated. Interestingly, the mobility shift on EndoH
digestion is intermediate between the undigested and the
PNGase F digested product, although all products are
discrete bands on a Western blot. This may suggest that
5   the two glycosylation sites are not identically modified
by the secretory pathway of the S2 cells. Estimates of
the expression level based upon Coomassie stained gel and
Western blot is ˜10 µg/ml.

        S2 DEN-2 NS1 transfectants were plated at
10   limiting dilutions in supplemented serum-free medium
(IPL-41; Life Technologies Inc.). The subcultures were
induced with $CuSO_4$ and the expression levels screened by
immuno-dot blot, using MAb 7E11 (NS1 specific) for
detection. Several of these cultures expressed and
15   secreted into the medium significant levels of NS1. The
highest level expressors will be expanded and a clonal
cell population secreting DEN-2 NS1 will be produced.

        A bioreactor culture of S2 DEN-2 NS1 cells was
grown using the *Drosophila* cell culture methods described
20   above. Based on Western dot blot analysis, we estimate
that approximately 3-4-fold more NS1 was produced per
unit volume in the bioreactor medium (˜10 µg/ml),
compared to S2 DEN-2 NS1 cultures grown in tissue culture
flasks. Analysis by SDS-PAGE of unpurified S2 DEN-2 NS1
25   culture medium, heated at 37 °C for 15 minutes in
nonreducing buffer, showed a distinct band migrating at
˜80-85 kDa, possibly corresponding to NS1 dimer. Western
blots employing the anti-NS1 specific MAb, 7E11,
confirmed that >90% of the NS1 secreted into culture
30   medium exists as a homodimer. SDS-PAGE/Western blot
analysis also indicated that S2 DEN-2 NS1 cells secreted
˜3-4—fold higher levels of NS1 dimer when cultured in
the bioreactor, compared to culture in flasks.

        For nondisruptive HPLC purification preserving
35   the dimer form a Hewlett-Packard Ti-series 1050 HPLC
system was employed, with UV detection at 280 nm.
Preliminary attempts with hydrophobic interaction

chromatography (TOSOHAAS phenyl-5PW) failed to resolve
any of the medium components. Thus, an ion exchange
method based on Cellufine sulfate (Amicon), was employed.
S2 DEN-2 NS1 containing medium from a bioreactor culture

5   was exchanged into 20 mM succinic acid/sodium succinate,
pH 6.0, and concentrated using a Minitan tangential flow
ultrafiltration unit (Millipore, Bedford, MA) equipped
with 10K molecular weight cut-off membranes. The
concentrate was applied to a Cellufine sulfate column

10 equilibrated with a running buffer of 20 mM succinic
acid/sodium succinate, pH 6.0, and the NS1 protein was
eluted with a linear 0--3M NaCl gradient developed over 5
column volumes. NS1 eluted in the 1--1.5 M NaCl fraction
and gave nearly complete recovery of ²50% pure NS1 dimer,

15 as judged by SDS-PAGE analysis.
          Further purification of the NS1 obtained in the
previous paragraph employed Fractogel DEAE (EM Sciences,
Gibbstown, NJ) which efficiently removes a major
Drosophila cell contaminant present in the Cellufine

20 sulfate fractions. Since the calculated isoelectric
point of NS1 is similar to that of 80%E, we initially
employed Fractogel DEAE column conditions that had been
developed for 80%. NS1 medium was exchanged into running
buffer, 20 mM Tris succinate, pH 8.1, containing 50 mM

25 NaCl, applied to Fractogel DEAE, then NS1 was eluted with
a NaCl step-gradient in pH 8.1 buffer. This protocol
resulted in ~50-75% pure NS1 dimer, as determined by SDS-
PAGE and Western blot.

<u>Claims</u>

1.   A vaccine for the protection of a subject
against flavivirus infection, which vaccine contains, as
5   active ingredient, a first envelope protein (E) or
subunit thereof from a flavivirus serotype, said E or
subunit having been secreted as a recombinantly produced
protein from eucaryotic cells.

10   2.   The vaccine of claim 1 which further
contains as active ingredient at least one second E or
subunit of the same or different flavivirus serotype,
said second E or subunit having been secreted as a mature
recombinantly produced protein from eucaryotic cells.
15

3.   The vaccine of claims 1 or 2 which further
contains as active ingredient at least one additional
flavivirus protein or subunit, other than E or subunit of
E, said additional flavivirus protein having been
20   secreted as a mature recombinantly produced protein from
eucaryotic cells.

4.   The vaccine of claim 3 wherein the
additional protein or subunit is NS1 or a subunit of NS-
25 1.

5.   The vaccine of any of claims 1-4 or
wherein the E subunit is 60%E, 80%E and/or B domain.

30   6.   The vaccine of any of claims 1-5 wherein
said eucaryotic cells are yeast cells and at
least one E or subunit or additional flavivirus protein
or subunit is encoded in a DNA construct operably linked
downstream from α-mating factor leader peptide (MFα$_L$); or
35   wherein said eucaryotic cells are *Drosophila*
cells and at least one E or subunit or additional
flavivirus protein or subunit, is encoded in a DNA

construct operably linked downstream from human tissue plasminogen activator prepropeptide secretion leader (tPA$_L$); or is operably linked downstream from a homologous prM sequence or C-terminal portion thereof containing a signal cleavage site or modified forms thereof.

7.   The vaccine of any of claims 1-6 which further contains an adjuvant.

8.   A method to protect a subject against flavivirus infection, which method comprises administering to a subject in need of such protection an effective amount of the vaccine of any of claims 1-7.

9.   The method of claim 8 which further includes administering a superattenuated form of flavivirus.

10.   A composition of antibodies consisting essentially of antibodies generated in a mammalian subject administered an immunogenic amount of the vaccine of any of claims 1-7.

11.   An immortalized B cell line, wherein said B cell has been generated in response to the administration of an immunogenic amount of the vaccine of any of claims 1-7 to a mammalian subject.

12.   Monoclonal antibodies secreted by the immortalized B cell line of claim 11.

13.   A passive vaccine for the protection of a subject against infection by the flavivirus, which vaccine contains, as active ingredient, the composition of claim 10 or the monoclonal antibodies of claim 12.

14. An expression system for the production of
mature flavivirus protein or a subunit thereof, which
expression system comprises a first nucleotide sequence
encoding said flavivirus protein or subunit and a second
5   encoding nucleotide sequence positioned so as to produce
a fusion protein wherein a secretory leader sequence is
operably linked to the flavivirus protein or subunit,
said encoding sequences operably linked to control
sequences capable of effecting expression of said
10   encoding nucleotide sequences.

15.   The expression system of claim 14 wherein
the flavivirus protein is the flavivirus envelope
protein.
15

16.   The expression system of claim 14 or 15
wherein the control sequences are nonviral.

17.   The expression system of any of claims 14-
20   16 wherein said secretory leader sequence is human tissue
plasminogen activator prepropeptide secretion leader
(tPA$_L$) or is yeast α-mating factor leader peptide (MFα$_L$)
or is flavivirus prM or a C-terminal protein thereof
which contains a signal sequence in native or modified
25   form.

18.   A eucaryotic host cell modified to contain
the expression system of any of claims 14-17.

30   19.   A method to produce a flavivirus protein
or a subunit thereof, which method comprises culturing
the cells of claim 18 under conditions favorable for
expression of the encoding nucleotide sequence so as to
secrete said flavivirus protein or subunit into the
35   medium.

20. A culture of eucaryotic cells wherein said cells contain a protein which consists of the flavivirus protein or a subunit thereof operably linked to a secretory leader sequence.

5

21. The cell culture of claim 20 wherein said flavivirus protein is E protein and the subunit is classical B domain, DomB+T, 60%E or 80%E and/or
   wherein said secretory leader sequence is yeast
10 α-mating factor leader peptide (MFα$_L$), human tissue plasminogen activator prepropeptide secretion leader (tPA$_L$), or flavivirus prM which contains a signal sequence in native or modified form.

FIG. I
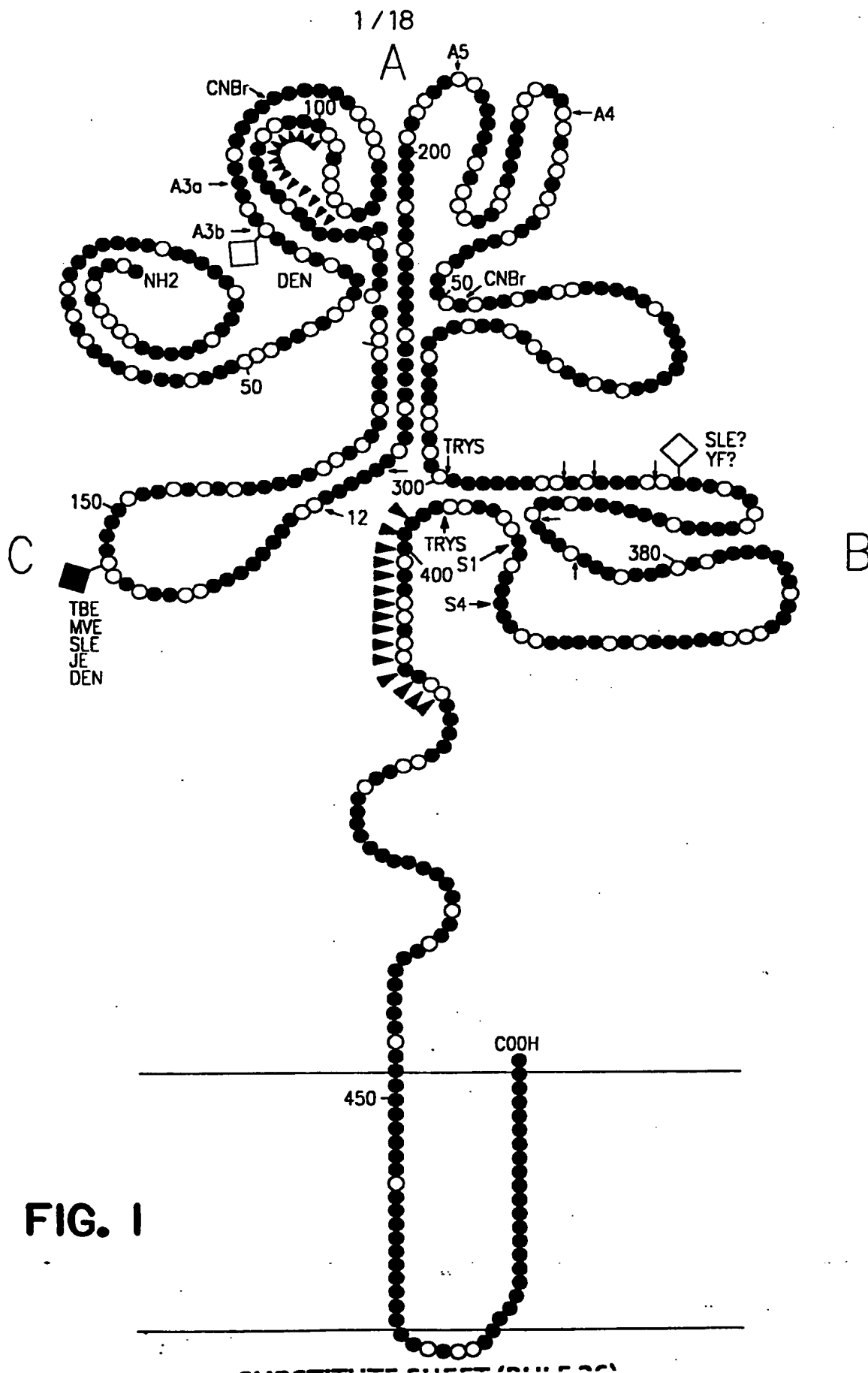
```
                    Capsid .
                       |         *
      97            ATGA ATAACCAACG GAAAAAGGCG AGAAACACGC CTTTCAATAT
     141  GCTGAAACGC GAGAGAAACC GCGTGTCAAC TGTACAACAG TTGACAAAGA
     191  GATTCTCACT TGGAATGCTG CAGGGACGAG GACCACTAAA ATTGTTCATG
     241  GCCCTGGTGG CATTCCTTCG TTTCCTAACA ATCCCACCAA CAGCAGGGAT
     291  ATTAAAAAGA TGGGGAACAA TTAAAAAATC AAAGGCTATT AATGTTCTGA
     341  GAGGCTTCAG GAAAGAGATT GGAAGGATGC TGAATATCTT AAACAGGAGA
                                                  preMembrane
                                                       |
     391  CGTAGAACTG CAGGCATGAT CATCATGCTG ATTCCAACAG TGATGGCGTT
     441  TCATCTGACC ACACGCAACG GAGAACCACA CATGATCGTC AGTAGACAAG
     491  AAAAAGGGAA AAGCCTTCTG TTTAAGACAA AGGACGGCAC GAACATGTGT
     541  ACCCTCATGG CCATGGACCT TGGTGAGTTG TGTGAAGACA CAATCACGTA
     591  TAAATGTCCC TTTCTCAAGC AGAACGAACC AGAAGACATA GATTGTTGGT
     641  GCAACTCCAC GTCCACATGG GTAACTTATG GGACATGTAC CACCACAGGA
                                  Membrane
                                     |
     691  GAGCACAGAA GAGAAAAAAG ATCAGTGGCG CTTGTTCCAC ACGTGGGAAT
     741  GGGATTGGAG ACACGAACTG AAACATGGAT GTCATCAGAA GGGGCCTGGA
     791  AACATGCCCA GAGAATTGAA ACTTGGATTC TGAGACATCC AGGCTTTACC
     841  ATAATGGCCG CAATCCTGGC ATACACCATA GGAACGACGC ATTTCCAAAG
                                                    Envelope
                                                       |
     891  AGTCCTGATA TTCATCCTAC TGACAGCCAT CGCTCCTTCA ATGACAATGC
     941  GCTGCATAGG AATATCAAAT AGGGACTTTG TGGAAGGAGT GTCAGGAGGG
     991  AGTTGGGTTG ACATAGTTTT AGAACATGGA AGTTGTGTGA CGACGATGGC
    1041  AAAAAATAAA CCAACACTGG ACTTTGAACT GATAAAAACA GAAGCCAAAC
    1091  AACCCGCCAC CTTAAGGAAG TACTGTATAG AGGCTAAACT GACCAACACG
    1141  ACAACAGACT CGCGCTGCCC AACACAAGGG GAACCCACCC TGAATGAAGA
    1191  GCAGGACAAA AGGTTTGTCT GCAAACATTC CATGGTAGAC AGAGGATGGG
    1241  GAAATGGATG TGGATTATTT GGAAAAGGAG GCATCGTGAC CTGTGCCATG
                                        A
    1291  TTCACATGCA AAAAGAACAT GGAGGGAAAA ATTGTGCAGC CAGAAAACCT
                              G
    1341  GGAATACACT GTCGTTATAA CACCTCATTC AGGGGAAGAA CATGCAGTCG
    1391  GAAATGACAC AGGAAAACAT GGTAAAGAAG TCAAGATAAC ACCACAGAGC
    1441  TCCATCACAG AGGCGGAACT GACAGGCTAT GGCACTGTTA CGATGGAGTG
    1491  CTCTCCAAGA ACGGGCCTCG ACTTCAATGA GATGGTGTTG CTGCAAATGA
    1541  AAGACAAAGC TTGGCTGGTG CACAGACAAT GGTTCCTAGA CCTACCGTTG
    1591  CCATGGCTGC CCGGAGCAGA CACACAAGGA TCAAATTGGA TACAGAAAGA
    1641  GACACTGGTC ACCTTCAAAA ATCCCCATGC GAAAAAACAG GATGTTGTTG
    1691  TCTTAGGATC CCAAGAGGGG GCCATGCATA CAGCACTCAC AGGGGCTACG
    1741  GAAATCCAGA TGTCATCAGG AAACCTGCTG TTCACAGGAC ATCTTAAGTG
    1791  CAGGCTGAGA ATGGACAAAT TACAACTTAA AGGGATGTCA TACTCCATGT
```

FIG. 2A

```
                              A
     1841  GCACAGGAAA GTTTAAAGTT GTGAAGGAAA TAGCAGAAAC ACAACATGGA
                                                                  *
     1891  ACAATAGTCA TTAGAGTACA ATATGAAGGA GACGGCTCTC CATGCAAGAT
     1941  CCCTTTTGAG ATAATGGATC TGGAAAAAAG ACATGTTTTG GGCCGCCTGA
           *                                     T*
     1991  TCACAGTCAA CCCAATTGTA ACAGAAAAGG ACAGCCCAGT CAACATAGAA
     2041  GCAGAACCTC CATTCGGAGA CAGCTACATC ATCATAGGAG TGGAACCAGG
     2091  ACAATTGAAG CTGGACTGGT TCAAGAAAGG AAGTTCCATC GGCCAAATGT
     2141  TTGAGACAAC AATGAGGGGA GCGAAAAGAA TGGCCATTTT GGGCGACACA
     2191  GCCTGGGATT TTGGATCTCT GGGAGGAGTG TTCACATCAA TAGGAAAGGC
     2241  TCTCCACCAG GTTTTTGGAG CAATCTACGG GGCTGCTTTC AGTGGGGTCT
     2291  CATGGACTAT GAAGATCCTC ATAGGAGTTA TCATCACATG GATAGGAATG
     2341  AACTCACGTA GCACATCACT GTCTGTGTCA CTGGTATTAG TGGGAATCGT
                                                       NS1
                         N                               |
     2391  GACACTGTAC TTGGGAGTTA TGGTGCAGGC CGATAGTGGT TGCGTTGTGA
     2441  GCTGGAAGAA CAAAGAACTA AAATGTGGCA GTGGAATATT CGTCACAGAT
     2491  AACGTGCATA CATGGACAGA ACAATACAAG TTCCAACCAG AATCCCCTTC
     2541  AAAACTGGCT TCAGCCATCC AGAAAGCTCA TGAAGAGGGC ATCTGTGGAA
     2591  TCCGCTCAGT AACAAGACTG GAAAATCTTA TGTGGAAACA AATAACATCA
     2641  GAATTGAATC ATATTCTATC AGAAAATGAA GTGAAACTGA CCATCATGAC
     2691  AGGAGACATC AAAGGAATCA TGCAGGTAGG AAAACGATCT CTGCGGCCTC
     2741  AACCCACTGA GTTGAGGTAT TCATGGAAAA CATGGGGTAA AGCGAAAATG
     2791  CTCTCCACAG AACTCCATAA TCAGACCTTC CTCATTGATG GTCCCGAAAC
     2841  AGCAGAATGC CCCAACACAA ACAGAGCTTG GAATTCACTA GAAGTTGAGG
     2891  ACTACGGCTT TGGAGTATTC ACTACCAATA TATGGCTAAG ATTGAGAGAA
     2941  AAGCAGGATG CATTTTGTGA CTCAAAACTC ATGTCAGCGG CCATAAAGGA
     2991  CAACAGAGCC GTCCATGCTG ATATGGGTTA TTGGATAGAA AGCGCACTCA
     3041  ATGATACATG GAAGATAGAG AAAGCTTCTT TCATTGAAGT CAAAAGTTGC
     3091  CACTGGCCAA AGTCACACAC TCTATGGAGT AATGGAGTGC TAGAAAGCGA
     3141  GATGGTAATT CCAAAGAATT TCGCTGGACC AGTGTCACAA CATAATAACA
     3191  GACCAGGCTA TCACACACAA ACAGCAGGAC CTTGGCATCT AGGCAAGCTT
     3241  GAGATGGACT TTGATTTCTG CGAAGGGACT ACAGTGGTGG TAACCGAGGA
     3291  CTGTGGAAAC AGAGGGCCCT CTTTAAGAAC AACCACTGCC TCAGGAAAAC
     3341  TCATAACGGA ATGGTGTTGT CGATCTTGCA CACTACCACC ACTAAGATAC
     3391  AGAGGTGAGG ATGGATGCTG GTACGGGATG·GAAATCAGAC CATTGAAAGA
     3441  GAAAGAAGAA AATCTGGTCA GTTCTCTGGT CACAGCC
```

DEN-2 PR159/S1 cDNA coding strand sequence for Capsid,
preMembrane, Envelope, and NS1 is given (Hahn et al. 1988).
The start of genes are indicated.
* indicates correction to published sequence
Differences in the wild-type DEN-2 PR159 sequence are
indicated above the PR159/S1 sequence.

FIG. 2B

```
 97                                    ATGA ATAACCAACG GAAAAAGGCG AGAAACACGC
                                       Met AsnAsnGlnArg LysLysAla ArgAsnThr>
                                       ● Capsid

131  CTTTCAATAT GCTGAAACGC GAGAGAAACC GCGTGTCAAC TGTACAACAG TTGACAAAGA
     ProPheAsnMet LeuLysArg GluArgAsn ArgValSerThr ValGlnGln LeuThrLys>

191  GATTCTCACT TGGAATGCTG CAGGGACGAG GACCACTAAA ATTGTTCATG GCCCTGGTGG
     ArgPheSerLeu GlyMetLeu GlnGlyArg GlyProLeuLys LeuPheMet AlaLeuVal>

251  CATTCCTTCG TTTCCTAACA ATCCCACCAA CAGCAGGGAT ATTAAAAAGA TGGGGAACAA
     AlaPheLeuArg PheLeuThr IleProPro ThrAlaGlyIle LeuLysArg TrpGlyThr>

311  TTAAAAAATC AAAGGCTATT AATGTTCTGA GAGGCTTCAG GAAAGAGATT GGAAGGATGC
     IleLysLysSer LysAlaIle AsnValLeu ArgGlyPheArg LysGluIle GlyArgMet>

371  TGAATATCTT AAACAGGAGA CGTAGAACTG CAGGCATGAT CATCATGCTG ATTCCAACAG
     LeuAsnIleLeu AsnArgArg ArgArgThr AlaGlyMetIle IleMetLeu IleProThr>

431  TGATGGCGTT TCATCTGACC ACACGCAACG GAGAACCACA CATGATCGTC AGTAGACAAG
     ValMetAlaPhe HisLeuThr ThrArgAsn GlyGluProHis MetIleVal SerArgGln>
                   ● PreMembrane

491  AAAAAGGGAA AAGCCTTCTG TTTAAGACAA AGGACGGCAC GAACATGTGT ACCCTCATGG
     GluLysGlyLys SerLeuLeu PheLysThr LysAspGlyThr AsnMetCys ThrLeuMet>

551  CCATGGACCT TGGTGAGTTG TGTGAAGACA CAATCACGTA TAAATGTCCC TTTCTCAAGC
     AlaMetAspLeu GlyGluLeu CysGluAsp ThrIleThrTyr LysCysPro PheLeuLys

611  AGAACGAACC AGAAGACATA GATTGTTGGT GCAACTCCAC GTCCACATGG GTAACTTATG
     GlnAsnGluPro GluAspIle AspCysTrp CysAsnSerThr SerThrTrp ValThrTyr>

671  GGACATGTAC CACCACAGGA GAGCACAGAA GAGAAAAAAG ATCAGTGGCG CTTGTTCCAC
     GlyThrCysThr ThrThrGly GluHisArg ArgGluLysArg SerValAla LeuValPro>
                                                   ● Membrane

731  ACGTGGGAAT GGGATTGGAG ACACGAACTG AAACATGGAT GTCATCAGAA GGGGCCTGGA
     HisValGlyMet GlyLeuGlu ThrArgThr GluThrTrpMet SerSerGlu GlyAlaTrp>

791  AACATGCCCA GAGAATTGAA ACTTGGATTC TGAGACATCC AGGCTTTACC ATAATGGCCG
     LysHisAlaGln ArgIleGlu ThrTrpIle LeuArgHisPro GlyPheThr IleMetAla>

851  CAATCCTGGC ATACACCATA GGAACGACGC ATTTCCAAAG AGTCCTGATA TTCATCCTAC
     AlaIleLeuAla TyrThrIle GlyThrThr HisPheGlnArg ValLeuIle PheIleLeu>
```

FIG. 3A

```
911   TGACAGCCAT CGCTCCTTCA ATGACAATGC GCTGCATAGG AATATCAAAT AGGGACTTTG
      LeuThrAlaIle AlaProSer MetThrMet ArgCysIleGly IleSerAsn ArgAspPhe>
                                        • Envelope

971   TGGAAGGAGT GTCAGGAGGG AGTTGGGTTG ACATAGTTTT AGAACATGGA AGTTGTGTGA
      ValGluGlyVal SerGlyGly SerTrpVal AspIleValLeu GluHisGly SerCysVal>

1031  CGACGATGGC AAAAAATAAA CCAACACTGG ACTTTGAACT GATAAAAACA GAAGCCAAAC
      ThrThrMetAla LysAsnLys ProThrLeu AspPheGluLeu IleLysThr GluAlaLys>

1091  AACCCGCCAC CTTAAGGAAG TACTGTATAG AGGCTAAACT GACCAACACG ACAACAGACT
      GlnProAlaThr LeuArgLys TyrCysIle GluAlaLysLeu ThrAsnThr ThrThrAsp>

1151  CGCGCTGCCC AACACAAGGG GAACCCACCC TGAATGAAGA GCAGGACAAA AGGTTTGTCT
      SerArgCysPro ThrGlnGly GluProThr LeuAsnGluGlu GlnAspLys ArgPheVal>

1211  GCAAACATTC CATGGTAGAC AGAGGATGGG GAAATGGATG TGGATTATTT GGAAAAGGAG
      CysLysHisSer MetValAsp ArgGlyTrp GlyAsnGlyCys GlyLeuPhe GlyLysGly>

                                                 GAA(Glu)
WT  1271  GCATCGTGAC CTGTGCCATG TTCACATGCA AAAAGAACAT GGAGGGAAAA ATTGTGCAGC
      GlyIleValThr CysAlaMet PheThrCys LysLysAsnMet GluGlyLys IleValGln>

                        GTG(Val)
WT  1331  CAGAAAACCT GGAATACACT GTCGTTATAA CACCTCATTC AGGGGAAGAA CATGCAGTCG
      ProGluAsnLeu GluTyrThr ValValIle ThrProHisSer GlyGluGlu HisAlaVal>

1391  GAAATGACAC AGGAAAACAT GGTAAAGAAG TCAAGATAAC ACCACAGAGC TCCATCACAG
      GlyAsnAspThr GlyLysHis GlyLysGlu ValLysIleThr ProGlnSer SerIleThr>

1451  AGGCGGAACT GACAGGCTAT GGCACTGTTA CGATGGAGTG CTCTCCAAGA ACGGGCCTCG
      GluAlaGluLeu ThrGlyTyr GlyThrVal ThrMetGluCys SerProArg ThrGlyLeu>

1511  ACTTCAATGA GATGGTGTTG CTGCAAATGA AAGACAAAGC TTGGCTGGTG CACAGACAAT
      AspPheAsnGlu MetValLeu LeuGlnMet LysAspLysAla TrpLeuVal HisArgGln>

1571  GGTTCCTAGA CCTACCGTTG CCATGGCTGC CCGGAGCAGA CACACAAGGA TCAAATTGGA
      TrpPheLeuAsp LeuProLeu ProTrpLeu ProGlyAlaAsp ThrGlnGly SerAsnTrp>

1631  TACAGAAAGA GACACTGGTC ACCTTCAAAA ATCCCCATGC GAAAAAACAG GATGTTGTTG
      IleGlnLysGlu ThrLeuVal ThrPheLys AsnProHisAla LysLysGln AspValVal>

1691  TCTTAGGATC CCAAGAGGGG GCCATGCATA CAGCACTCAC AGGGGCTACG GAAATCCAGA
      ValLeuGlySer GlnGluGly AlaMetHis ThrAlaLeuThr GlyAlaThr GluIleGln>

1751  TGTCATCAGG AAACCTGCTG TTCACAGGAC ATCTTAAGTG CAGGCTGAGA ATGGACAAAT
      MetSerSerGly AsnLeuLeu PheThrGly HisLeuLysCys ArgLeuArg MetAspLys>
```

FIG. 3B

```
                                                                    ATT(Ile)
WT 1811  TACAACTTAA AGGGATGTCA TACTCCATGT GCACAGGAAA GTTTAAAGTT GTGAAGGAAA
         LeuGlnLeuLys GlyMetSer TyrSerMet CysThrGlyLys PheLysVal ValLysGlu>

   1871  TAGCAGAAAC ACAACATGGA ACAATAGTCA TTAGAGTACA ATATGAAGGA GACGGCTCTC
         IleAlaGluThr GlnHisGly ThrIleVal IleArgValGln TyrGluGly AspGlySer>

   1931  CATGCAAGAT LCCTTTTGAG ATAATGGATC TGGAAAAAAG ACATGTTTTG GGCCGCCTGA
         ProCysLysIle ProPheGlu IleMetAsp LeuGluLysArg HisValLeu GlyArgLeu>

WT 1991  TCACAGTCAA CCCAATTGTA ACAGAAAAGG ACAGCCCAGT CAACATAGAA GCAGAACCTC
         IleThrValAsn ProIleVal ThrGluLys AspSerProVal AsnIleGlu AlaGluPro>

   2051  CATTCGGAGA CAGCTACATC ATCATAGGAG TGGAACCAGG ACAATTGAAG CTGGACTGGT
         ProPheGly Sp SerTyrIle IleIleGly ValGluProGly GlnLeuLys LeuAspTrp>

   2111  TCAAGAAAGG AAGTTCCATC GGCCAAATGT TTGAGACAAC AATGAGGGGA GCGAAAAGAA
         PheLysLysGly SerSerIle GlyGlnMet PheGluThrThr MetArgGly AlaLysArg>

   2171  TGGCCATTTT GGGCGACACA GCCTGGGATT TTGGATCTCT GGGAGGAGTG TTCACATCAA
         MetAlaIleLeu GlyAspThr AlaTrpAsp PheGlySerLeu GlyGlyVal PheThrSer>

   2231  TAGGAAAGGC TCTCCACCAG GTTTTTGGAG CAATCTACGG GGCTGCTTTC AGTGGGGTCT
         IleGlyLysAla LeuHisGln ValPheGly AlaIleTyrGly AlaAlaPhe SerGlyVal>

   2291  CATGGACTAT GAAGATCCTC ATAGGAGTTA TCATCACATG GATAGGAATG AACTCACGTA
         SerTrpThrMet LysIleLeu IleGlyVal IleIleThrTrp IleGlyMet AsnSerArg>

   2351  GCACATCACT GTCTGTGTCA CTGGTATTAG TGGGAATCGT GACACTGTAC TTGGGAGTTA
         SerThrSerLeu SerValSer LeuValLeu ValGlyIleVal ThrLeuTyr LeuGlyVal>

   2411  TGGTGCAGGC CGATAGTGGT TGCGTTGTGA GCTGGAAGAA CAAAGAACTA AAATGTGGCA
         MetValGlnAla AspSerGly CysValVal SerTrpLysAsn LysGluLeu LysCysGly>
                        ◆ NS1

   2471  GTGGAATATT CGTCACAGAT AACGTGCATA CATGGACAGA ACAATACAAG TTCCAACCAG
         SerGlyIlePhe ValThrAsp AsnValHis ThrTrpThrGlu GlnTyrLys PheGlnPro>

   2531  AATCCCCTTC AAAACTGGCT TCAGCCATCC AGAAAGCTCA TGAAGAGGGC ATCTGTGGAA
         GluSerProSer LysLeuAla SerAlaIle GlnLysAlaHis GluGluGly IleCysGly>

   2591  TCCGCTCAGT AACAAGACTG GAAAATCTTA TGTGGAAACA AATAACATCA GAATTGAATC
         IleArgSerVal ThrArgLeu GluAsnLeu MetTrpLysGln IleThrSer GluLeuAsn>

   2651  ATATTCTATC AGAAAATGAA GTGAAACTGA CCATCATGAC AGGAGACATC AAAGGAATCA
         HisIleLeuSer GluAsnGlu ValLysLeu ThrIleMetThr GlyAspIle LysGlyIle>

   2711  TGCAGGTAGG AAAACGATCT CTGCGGCCTC AACCCACTGA GTTGAGGTAT TCATGGAAAA
         MetGlnValGly LysArgSer LeuArgPro GlnProThrGlu LeuArgTyr SerTrpLys>
```

FIG. 3C

2771 CATGGGGTAA AGCGAAAATG CTCTCCACAG AACTCCATAA TCAGACCTTC CTCATTGATG
     ThrTrpGlyLys AlaLysMet LeuSerThr GluLeuHisAsn GlnThrPhe LeuIleAsp>

2831 GTCCCGAAAC AGCAGAATGC CCCAACACAA ACAGAGCTTG GAATTCACTA GAAGTTGAGG
     GlyProGluThr AlaGluCys ProAsnThr AsnArgAlaTrp AsnSerLeu GluValGlu>

2891 ACTACGGCTT TGGAGTATTC ACTACCAATA TATGGCTAAG ATTGAGAGAA AAGCAGGATG
     AspTyrGlyPhe GlyValPhe ThrThrAsn IleTrpLeuArg LeuArgGlu LysGlnAsp>

2951 CATTTTGTGA CTCAAAACTC ATGTCAGCGG CCATAAAGGA CAACAGAGCC GTCCATGCTG
     AlaPheCysAsp SerLysLeu MetSerAla AlaIleLysAsp AsnArgAla ValHisAla>

3011 ATATGGGTTA TTGGATAGAA AGCGCACTCA ATGATACATG GAAGATAGAG AAAGCTTCTT
     AspMetGlyTyr TrpIleGlu SerAlaLeu AsnAspThrTrp LysIleGlu LysAlaSer>

3071 TCATTGAAGT CAAAAGTTGC CACTGGCCAA AGTCACACAC TCTATGGAGT AATGGAGTGC
     PheIleGluVal LysSerCys HisTrpPro LysSerHisThr LeuTrpSer AsnGlyVal>

3131 TAGAAAGCGA GATGGTAATT CCAAAGAATT TCGCTGGACC AGTGTCACAA CATAATAACA
     LeuGluSerGlu MetValIle ProLysAsn PheAlaGlyPro ValSerGln HisAsnAsn>

3191 GACCAGGCTA TCACACACAA ACAGCAGGAC CTTGGCATCT AGGCAAGCTT GAGATGGACT
     ArgProGlyTyr HisThrGln ThrAlaGly ProTrpHisLeu GlyLysLeu GLuMetAsp>

3251 TTGATTTCTG CGAAGGGACT ACAGTGGTGG TAACCGAGGA CTGTGGAAAC AGAGGGCCCT
     PheAspPheCys GluGlyThr ThrValVal ValThrGluAsp CysGlyAsn ArgGlyPro>

3311 CTTTAAGAAC AACCACTGCC TCAGGAAAAC TCATAACGGA ATGGTGTTGT CGATCTTGCA
     SerLeuArgThr ThrThrAla SerGlyLys LeuIleThrGlu TrpCysCys ArgSerCys>

3371 CACTACCACC ACTAAGATAC AGAGGTGAGG ATGGATGCTG GTACGGGATG GAAATCAGAC
     ThrLeuProPro LeuArgTyr ArgGlyGlu AspGlyCysTrp TyrGlyMet GluIleArg>

3431 CATTGAAAGA GAAAGAAGAA AATCTGGTCA GTTCTCTGGT CACAGCC
     ProLeuLysGlu LysGluGlu AsnLeuVal SerSerLeuVal ThrAla

FIG. 3D

Oligonucleotide Mutagenesis to Generate
60%E and Domain B

Before mutagenesis:
1789   Trypsin   1848
  | ↓ |
5'-TGCAGGCTGAGAATGGACAAATTACAACTTAAAGGGATGTCATACTCCATGTGCACAGGA-3'
 CysArgLeuArgMetAspLysGlnLeuLysGlyMetSerTyrSerMetCysThrGly

Mutagenizing Oligonucleotide:
3'-CCGACTCTTACCTGTTA   CCCTACAGTATGAGG-5'
            \   /
          ctatcagctgtcga

After mutagenesis:
                SalI   PvuII
                 ∨   ∨
5'-TGCAGGCTGAGAATGGACAAATgatagtcgacagctGGGATGTCATACTCCATGTGCACAGGA-3'
 CysArgLeuArgMetAspLysEndEnd   GlyMetSerTyrSerMetCysThrGly...
    C-terminus of 60%E   N-terminus of domain B

Shown are the nucleotide sequence and the corresponding translation of
DEN-2 PR159/S1 E sequences between genomic nucleotides 1789 and 1848,
the sequence of the oligonucleotide used in mutagenesis, and the resulting
sequence and corresponding translations following mutagenesis. Dengue
nucleotide sequence is uppercase, non-dengue nucleotides are lower case,
inserted restriction endonuclease sites are indicated, and conserved
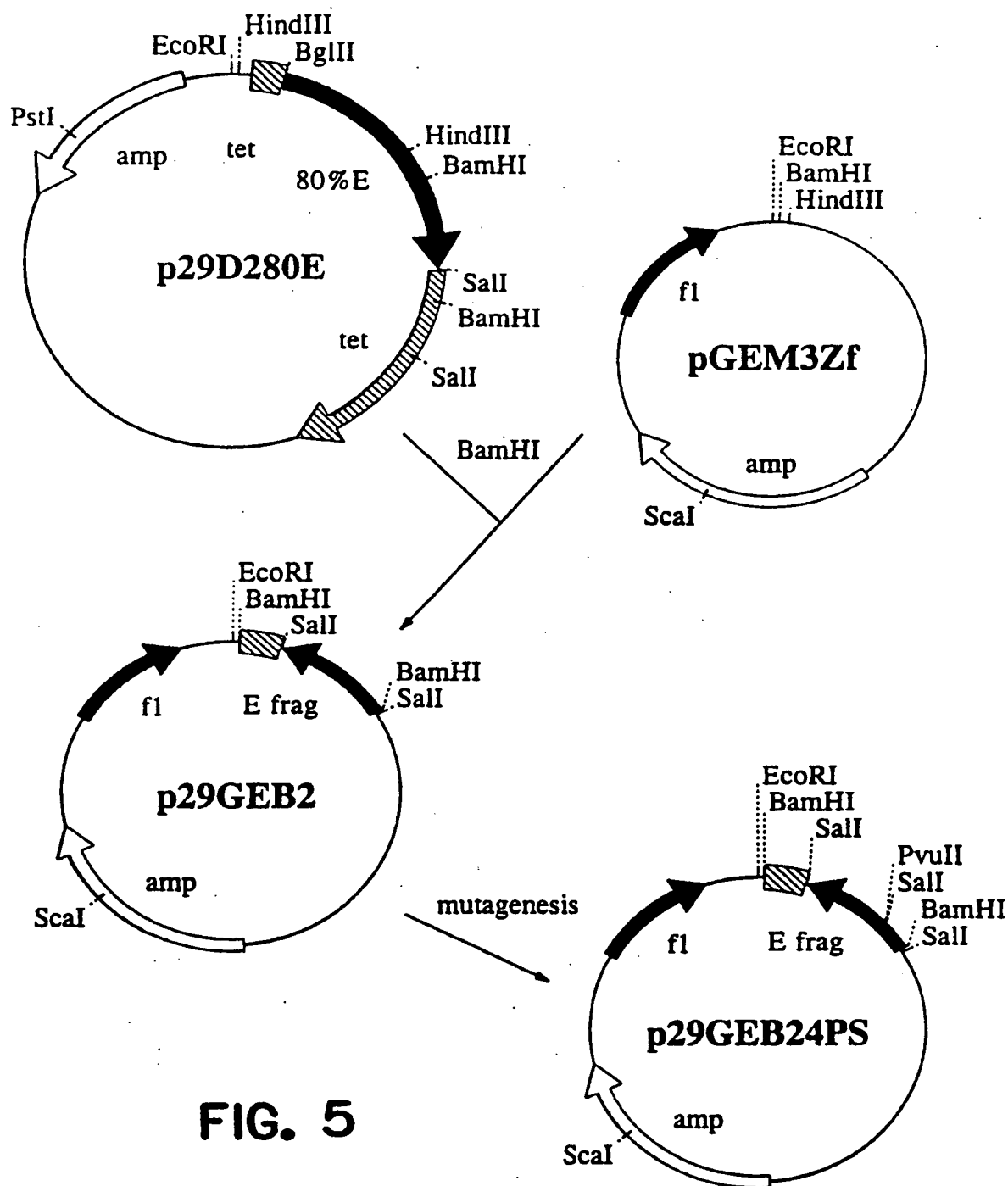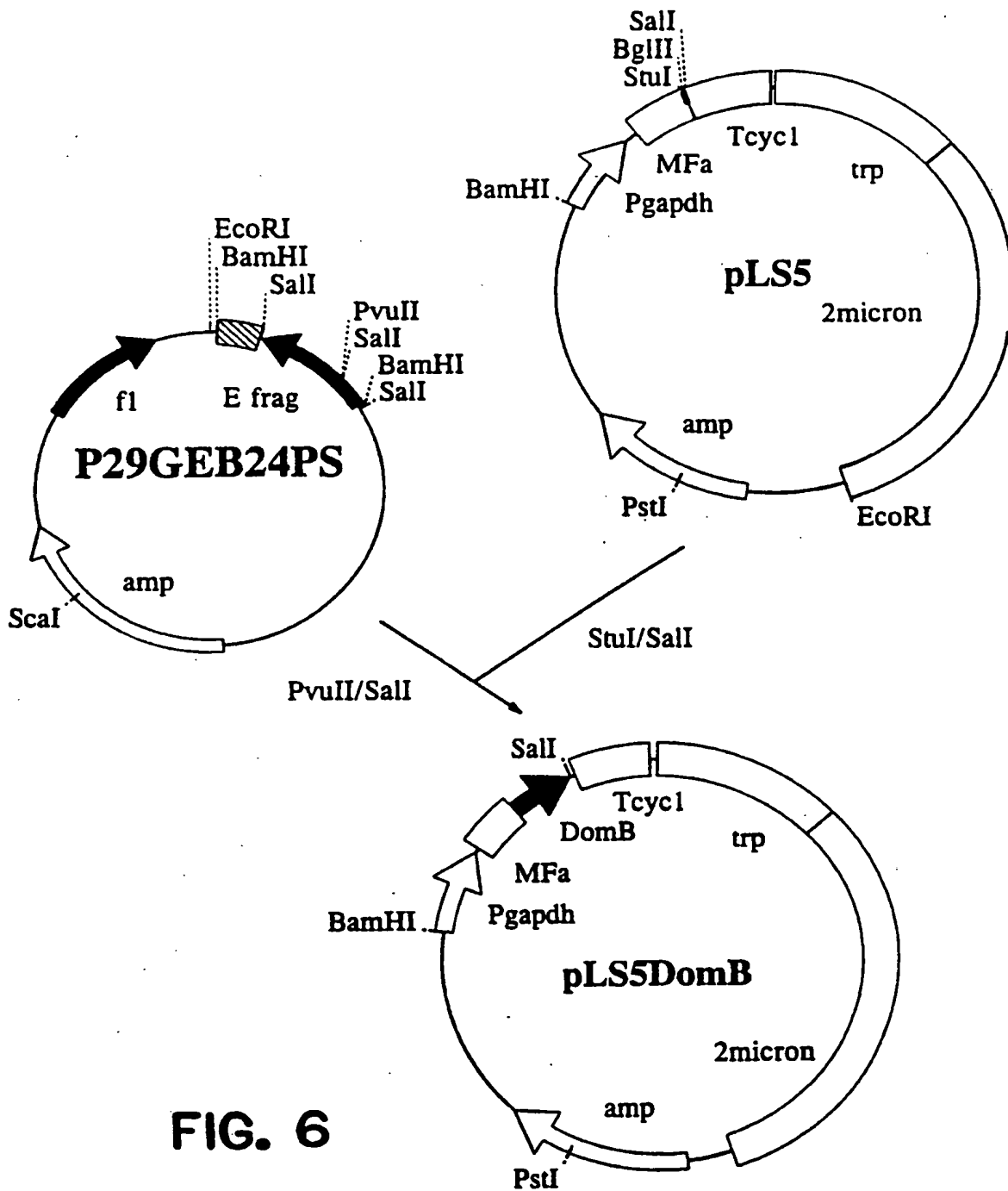cysteines are underlined.

FIG. 4

FIG. 5

FIG. 6

5318 ATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGC
      MetArgPheProSerIlePheThrAlaValLeuPheAlaAlaSerSerAla>
      ⊢▪MFα secretion signal peptide

  5368 ATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTC
        LeuAlaAlaProValAsnThrThrThrGluAspGluThrAlaGlnIle>
                  ▲ Signalase cleavage
                  ⊢▪MFα propeptide

  5418 CGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT
        ProAlaGluAlaValIleGlyTyrSerAspLeuGluGlyAspPheAspVal>

  5468 GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAA
        AlaValLeuProPheSerAsnSerThrAsnAsnGlyLeuLeuPheIleAsn>

  5518 TACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGA
        ThrThrIleAlaSerIleAlaAlaLysGlnGluGlyValSerLeuGlu>

  5568 AAAGGGAGGCTGGGATGTCATACTCCATGTGCACAGGAAAGTTTAAAGTT
        LysArgGluAlaGlyMetSerTyrSerMetCysThrGlyLysPheLysVal>
                  ▲ Kex2p cleavage
                    ⊢▪Domain B

  5618 GTGAAGGAAATAGCAGAAACACAACATGGAACAATAGTCATTAGAGTACA
        ValLysGluIleAlaGluThrGlnHisGlyThrIleValIleArgValGln>

  5668 ATATGAAGGAGACGGCTCTCCATGCAAGATCCCTTTTGAGATAATGGATC
        TyrGluGlyAspGlySerProCysLysIleProPheGluIleMetAsp>

  5718 TGGAAAAAAGACATGTTTTGGGCCGCCTGATCACAGTCAATCCAATTGTA
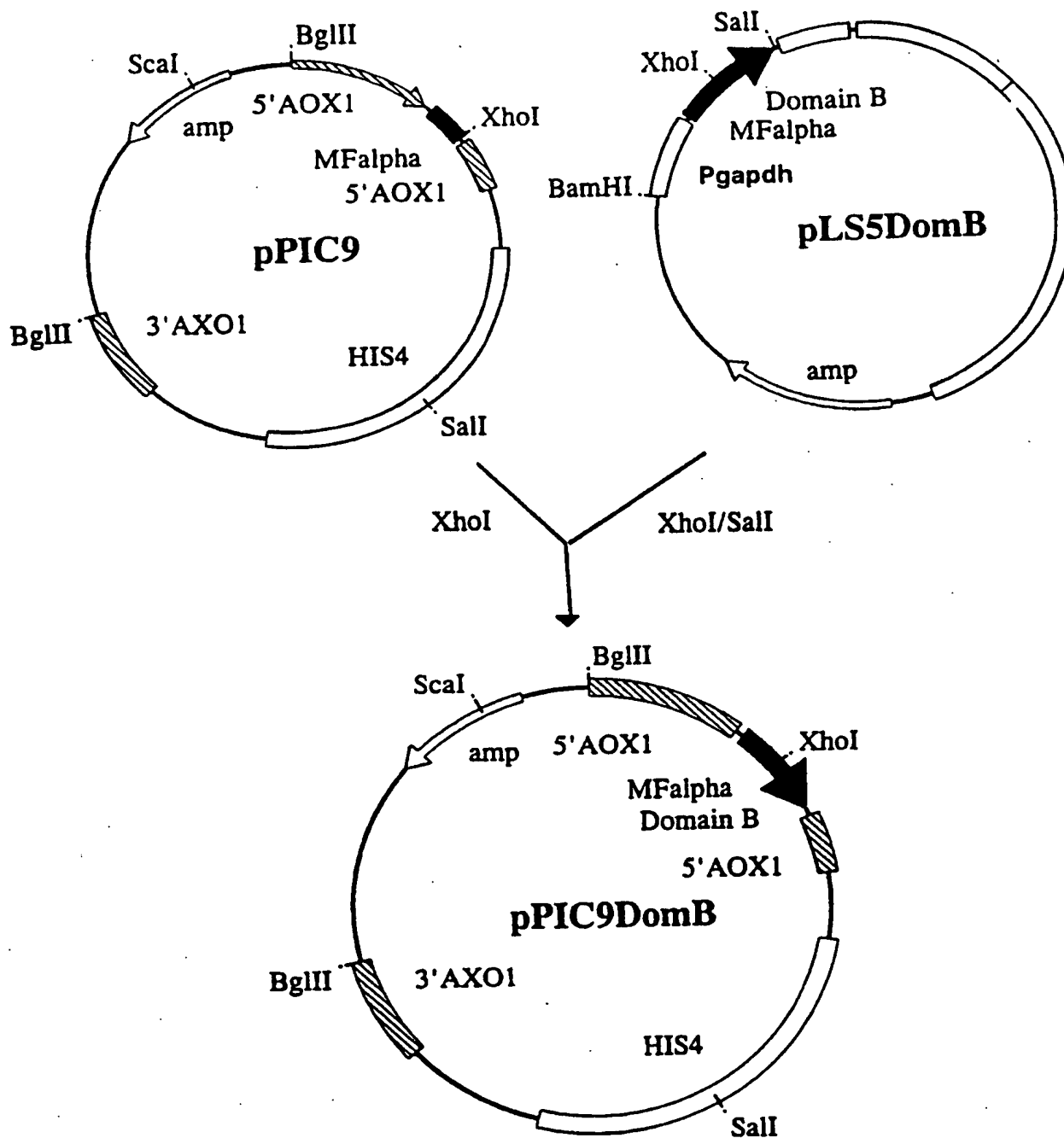   LeuGluLysArgHisValLeuGlyArgLeuIleThrValAsnProIleVal>

  5768 ACAGAAAAGGACAGCCCAGTCAACATAGAAGCAGAACCTCCATTCGGAGA
        ThrGluLysAspSerProValAsnIleGluAlaGluProProPheGlyAsp>

  5818 CAGCTACATCATCATAGGAGTGGAACCAGGACAATTGAAGCTGGACTGGT
        SerTyrIleIleIleGlyValGluProGlyGlnLeuLysLeuAspTrp>

  5868 TCAAGAAAGGATAA
        PheLysLysGlyEnd>

FIG. 7

FIG. 8

LOCUS   MTTDOMB   417   BP DS-DNA   ENTERED   12/05/94
DEFINITION   Coding sequence of tPA-domain B fusion protein
ACCESSION   -
KEYWORDS   -
SOURCE   -
ORGANISM   -
FEATURES   From To/Span   Description
   sigp   1   60   1 tPA Secretion Signal
   pept   61   93   1 tPA Propeptide
   matp   94   417   1 RGARSP-domB
   site   112   112   1 Domain B
BASE COUNT   138 A   85 C   106 G   88 T   0 OTHER
ORIGIN   -

         10   20   30   40   50   60   70
          *   *   *   *   *   *   *
ATGGATGCAA TGAAGAGAGG GCTCTGCTGT GTGCTGCTGC TGTGTGGAGC AGTCTTCGTT TCGCCCAGCC
TACCTACGTT ACTTCTCTCC CGAGACGACA CACGACGACG ACACACCTCG TCAGAAGCAA AGCGGGGTCGG
MetAspAla MetLysArgGly LeuCysCys ValLeuLeu LeuCysGlyAla ValPheVal>
          a   a   >SerProSer>
                 TPA SECRETION SIGNAL_a

         80   90   100   110   120   130   140
          *   *   *   *   *   *   *
AGGAAATCCA TGCCCGATTC AGAAGAGGAG CCAGATCCCC TGGGATGTCA TACTCCATGT GCACAGGAAA
TCCTTTAGGT ACGGGCTAAG TCTTCTCCTC GGTCTAGGGG ACCCTACAGT ATGAGGTACA CGTGTCCTTT
GlnGluIleHis AlaArgPhe Arg>   ArgGly AlaArgSerPro GlyMetSer TyrSerMet CysThrGlyLys>
          TPA PROPEPTIDE_b   c   c>
                                         >Domain_B
                                              ^ | >
                                              RGARSP-DOMB

FIG. 9A

```
        150       160       170        180       190        200       210
         *         *         *          *         *          *         *
GTTAAAATT  GTGAAGGAAA TAGCAGAAAC ACAACATGGA ACAATAGTCA TTAGAGTACA ATATGAAGGA
CAAATTTAA  CACTTCCTTT ATCGTCTTTG TGTTGTACCT TGTTATCAGT AATCTCATGT TATACTTCCT
PheLysIle ValLysGlu IleAlaGluThr GlnHisGly ThrIleVal IleArgValGln TyrGluGly>
RGARSP-DOMB_____^
                                                                      C

        220       230       240        250       260        270       280
         *         *         *          *         *          *         *
GACGGCTCTC CATGCAAGAT CCCTTTGAG ATAATGGATC TGGAAAAAAG ACATGTTTTG GGCCGCCTGA
CTGCCGAGAG GTACGTTCTA GGGAAAACTC TATTACCTAG ACCTTTTTTC TGTACAAAAC CCGGCGGACT
AspGlySer ProCysLysIle ProPheGlu IleMetAsp LeuGluLysArg HisValLeu GlyArgLeu>
RGARSP-DOMB_____^
                                                                     C

        290       300       310        320       330        340       350
         *         *         *          *         *          *         *
TCACAGTCAA CCCAATTGTA ACAGAAAAGG ACAGTCCAGT CAACATAGAA GCAGAACCTC CATTCGGAGA
AGTGTCAGTT GGGTTAACAT TGTCTTTTCC TGTCAGGTCA GTTGTATCTT CGTCTTGGAG GTAAGCCTCT
IleThrValAsn ProIleVal ThrGluLys AspSerProVal AsnIleGlu AlaGluPro ProPheGlyAsp>
RGARSP-DOMB_____^
                                                                     C

        360       370       380        390       400        410
         *         *         *          *         *          *
CAGCTACATC ATCATAGGAG TGGAACCAGG ACAATTGAAG CTGGACTGGT TCAAGAAAGG ATAATAG
GTCGATGTAG TAGTATCCTC ACCTTGGTCC TGTTAACTTC GACCTGACCA AGTTCTTTCC TATTATC
SerTyrIle IleIleGly ValGluProGly GlnLeuLys LeuAspTrp PheLysLysGly EndEnd>
RGARSP-DOMB_____^
                                                                    C
```
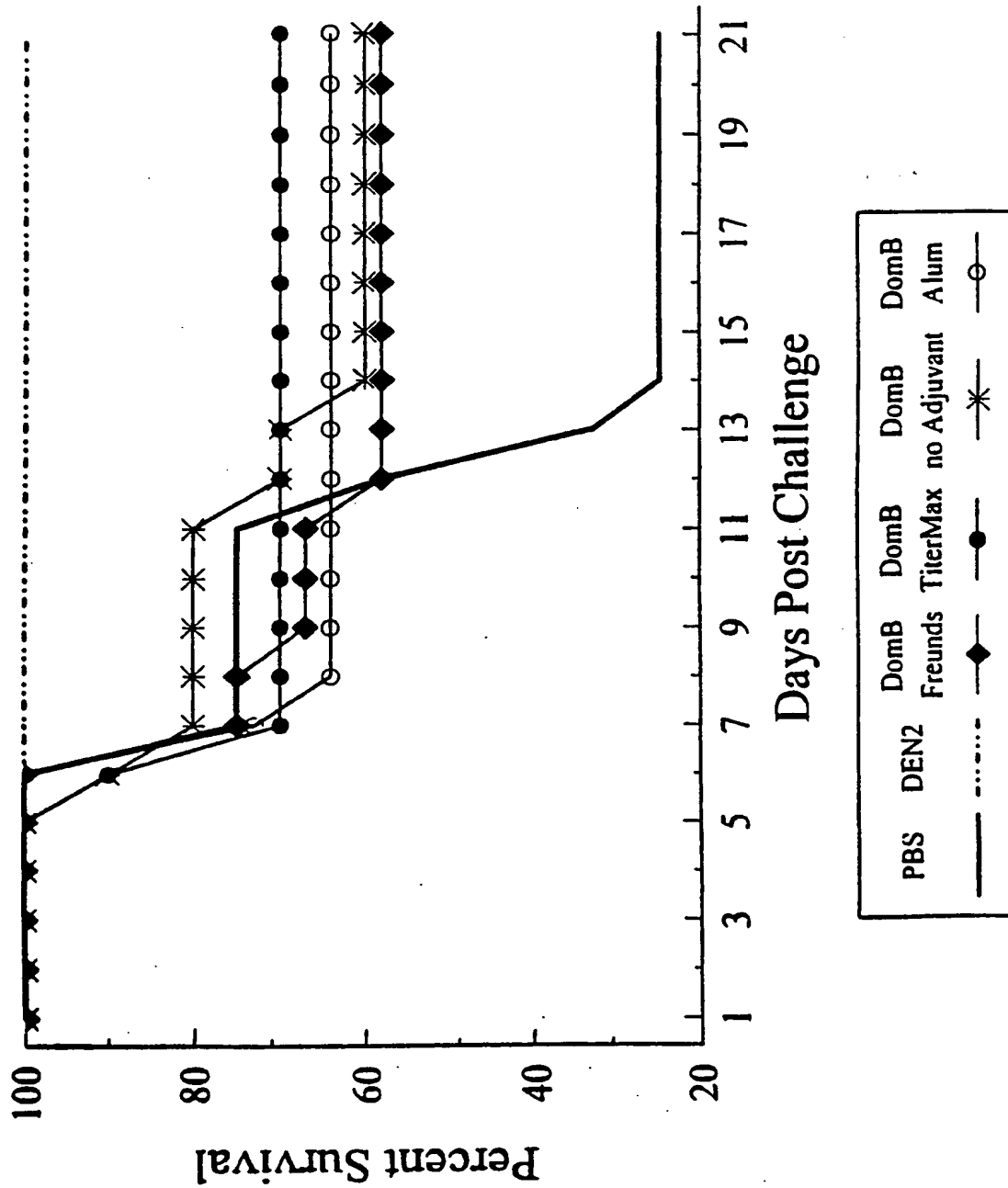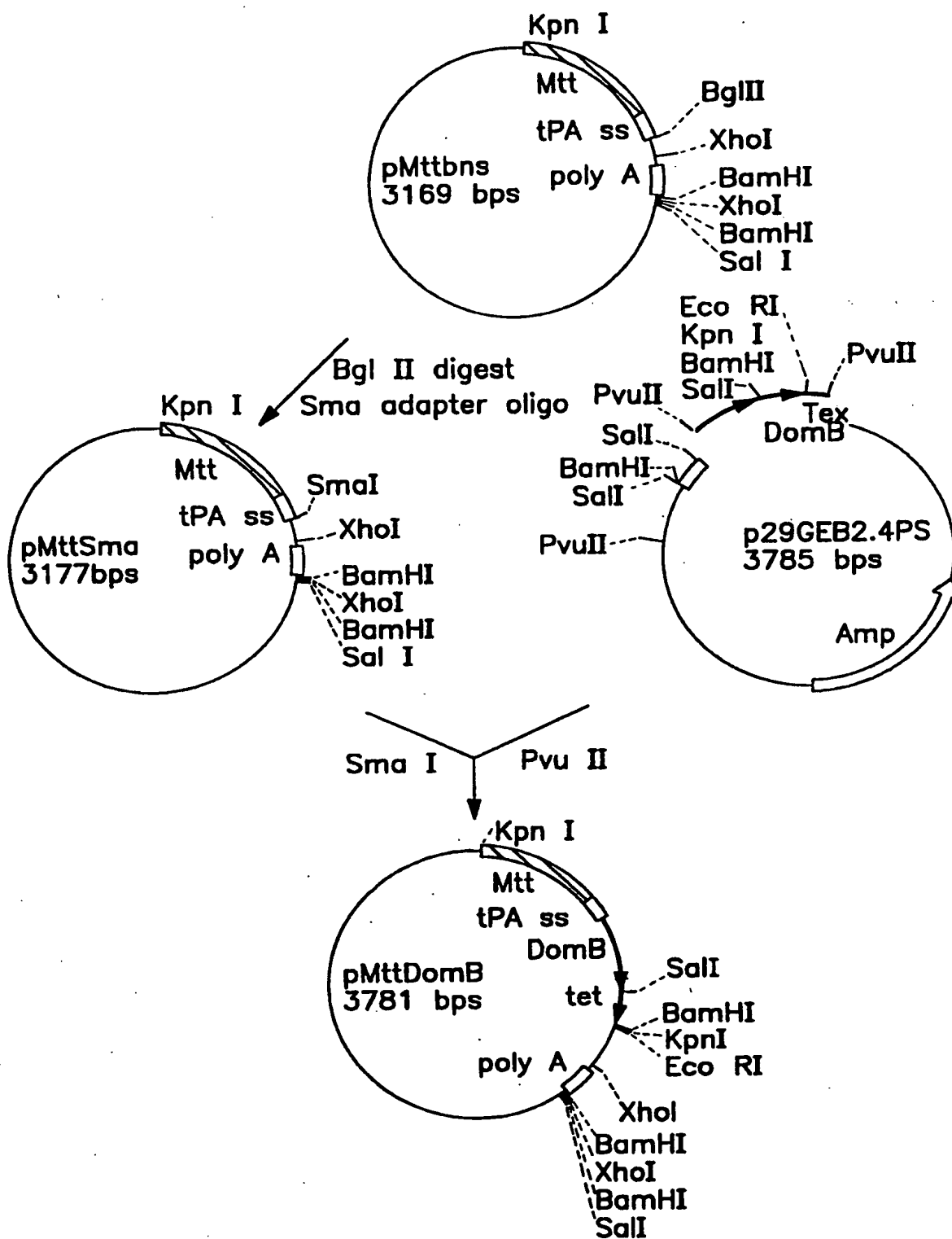
FIG. 9B

FIG. 10

FIG. II
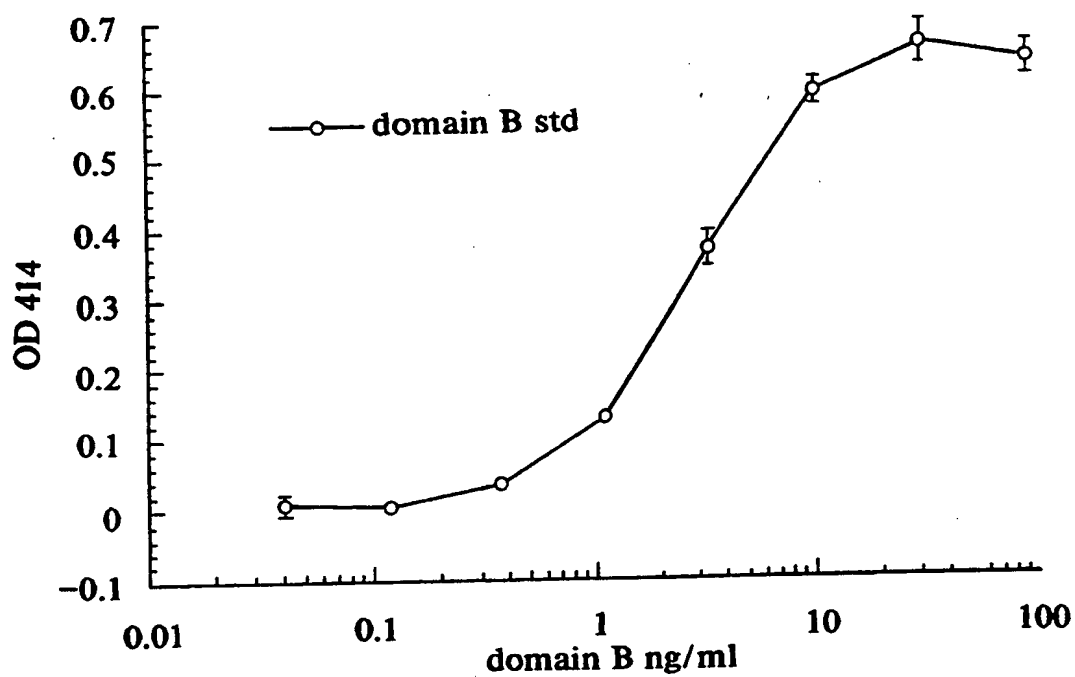
FIG. 12
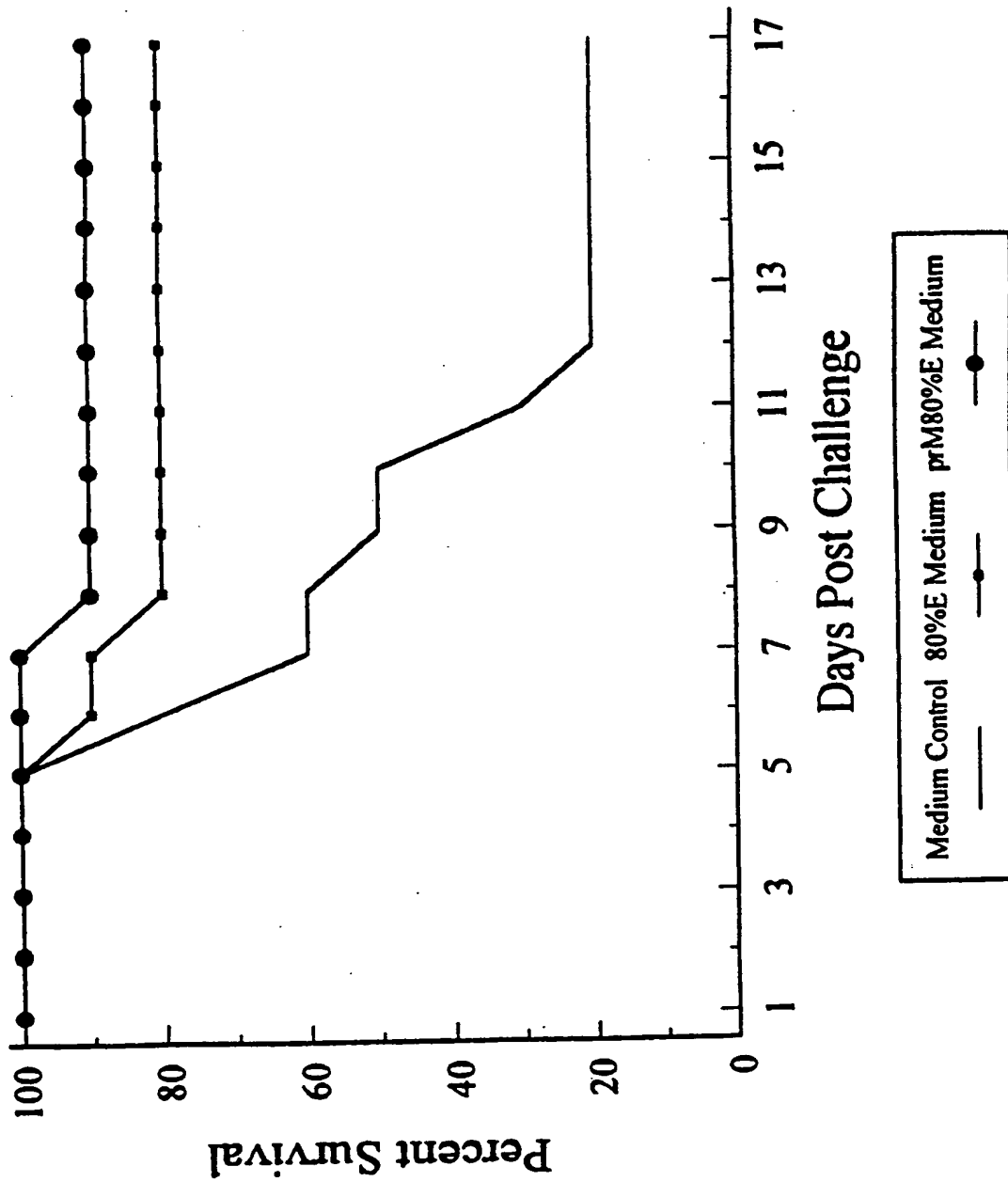
FIG. 13

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :424/218.1, 202.1; 536/23.72; 530/300, 350; 435/69.1, 252.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/218.1, 202.1; 536/23.72; 530/300, 350; 435/69.1, 252.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, WPIDS, DIALOG
search terms: dengue virus, envelop protein, vaccine, antibodies

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US, A, 5,494,671 (LAI ET AL) 27 February 1996, column 6, lines 36-65 and column 18, lines 27-40.	1-4, 14-16 and 20-21
X, P	US, A, 5,514,375 (PAOLETTI ET AL) 07 May 1996, see Figure 19.	1-4, 14-16 and 20-21
X	Arch. Virol, Volume 139, issued 1994, Delenda et al, "Protective efficacy in mice of a secreted form of recombinant dengue-2 virus envelope protein produced in baculovirus infected insect cells", pages 197-207, see pages 199-200.	1-4, 14-16 and 20-21

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 JULY 1996	27 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JULIE REEVES
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/12, 39/193, 39/295; C07H 21/04; C07K 14/18; C12P 21/06; C12N 1/20